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14. ABSTRACT

Several methods for diagnosis of exposure to chemical warfare agents and other noxious compounds have been transferred to Centers for Disease
Control & Prevention.
1. The modified Edman degradation of sulfur mustard-modified hemoglobin
2. The method for diagnosis of exposure to Lewisite
3. The method for diagnosis of exposure to phosgene
In addition, a number of existing methods have been improved:
4. The albumin tripeptide assay for diagnosis of exposure to sulfur mustard
5. The rapid assay for determination of exposure to nerve agents, based on pepsin digestion of human butyrylcholinesterase
In order to circumvent the shortcomings of the current (specific) assays for diagnosis of exposure to OP-anticholinesterases, a generic method for
detection of covalently modified human butyrylcholinesterase has been undertaken. Finally, various reference compounds have been synthesized and
delivered to CDC.

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PI - Signature Date

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SUMMARY

Several methods for diagnosis of exposure to chemical warfare agents and other toxic compounds have been transferred to Centers for Disease Control & Prevention.

1. The modified Edman degradation of sulfur mustard-modified hemoglobin
2. The method for diagnosis of exposure to Lewisite
3. The method for diagnosis of exposure to phosgene

A number of existing methods have been improved:

- The albumin tripeptide assay for diagnosis of exposure to sulfur mustard
- The rapid assay for determination of exposure to nerve agents, based on pepsin digestion of human butyrylcholinesterase (HuBuChE)

In order to circumvent the shortcomings of the current (specific) assays for diagnosis of exposure to OP-anticholinesterases, the development of a generic method for detection of covalently modified HuBuChE has been undertaken. Finally, various reference compounds have been synthesized and delivered to CDC.

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I INTRODUCTION

The work described in this annual report has been performed by the direction of Centers for Disease Control and Prevention (CDC), as an extension of cooperative agreement DAMD 17-02-2-0012.
The work is focused on

1. Transfer of methods for diagnosis of exposure to chemical warfare agents and toxic scheduled chemicals
2. Improvement of existing assays
3. Development of a general method for detection of covalent cholinesterase inhibitors
4. Development of assays to detect exposure to other toxic chemicals, likely to be used for chemical terrorism

Ad 1. Transfer of methods

We have proposed that two methods will be transferred to CDC: the method for diagnosis of lewisite exposure (Fidder et al., 2000) and an alternative method for diagnosis of sulfur mustard exposure, i.e., the modified Edman degradation of sulfur mustard-modified hemoglobin (Fidder et al., 1996; Noort et al., 2004). In more detail:

a. Protocols for the modified Edman degradation and the method for diagnosis of Lewisite exposure will be delivered to CDC. The procedures will be demonstrated at CDC by TNO employees.
b. The following reference materials will be delivered to CDC:
   - frozen blood samples (erythrocytes; 5 ml) which have been exposed to various (three) concentrations of Lewisite
   - the reference compound lewisite – British Antilewisite complex (5x 1 mg)
   - internal standard phenylarsineoxide – British Antilewisite complex (5x 1 mg)
   - pentafluorophenyl thiohydantoin of the N-terminal D/L-valine sulfur mustard adduct (1 mg)
   - globin samples (100 mg), isolated from blood which has been exposed to three different concentrations of sulfur mustard
   - globin, isolated from human blood which had been exposed to d_8-sulfur mustard (10 µM; 100 mg).

Ad 2. Improvement of existing assays

a. Sulfur mustard adducts

We propose to attempt to automate the albumin-tripeptide assay. The bottleneck of the albumin – tripeptide assay has been the laborious and lengthy (overnight!) isolation procedure for albumin from human plasma. Recently, TNO-PML developed a rapid isolation procedure for albumin from human plasma based on affinity chromatography, which takes only 30 min. In combination with a column of immobilized pronase, this might enable the development of a fully automated analytical system. Examples of such systems with other digestion enzymes have been reported in the literature (Hsieh et al, 1996; Nadler et al., 1996) and could also be applied for protein adduct determination (Shen et al, 2000). The advantages of such an automated system is that it reduces the reaction time, minimizes sample loss, improves cleavage efficiency and affords more reproducible digests. It is envisaged that the developed methodology will enable processing of large numbers of human blood samples. First, it will be investigated whether immobilized pronase gives rise to the formation of the adducted tripeptide as well.

Expected deliverables
- a methodology for rapid isolation of albumin from human plasma
- a methodology for albumin digestion on a column of immobilized pronase
- implementation of the two abovementioned methodologies into an automated system

b. Rapid assays for nerve agent adducts
In addition to the fluoride reactivation method, we recently developed an alternative mass spectrometric assay for diagnosis of exposure to nerve agents, which is based on LC-tandem MS analysis of a phosphylated nonapeptide in a pepsin digest of human butyrylcholinesterase (HuBuChE; Fidder et al., 2002), *i.e.*, one of the major targets of nerve agents. The advantages of this method are that it bypasses the use of highly toxic analytical standards and that it can be used for a wide variety of agents, including those that suffer from aging, a process which precludes the use of the fluoride reactivation method.
In case of screening of large numbers of human blood samples for assessment of exposure to nerve agents, a procedure for automated isolation and digestion of phosphylated HuBuChE, *i.e.*, the biomarker of interest, is highly desirable. In the current assay, isolation of HuBuChE is performed by affinity chromatography on a column with procainamide gel; the gel is prepared in our laboratory. However, this isolation procedure is quite laborious. Although we made some advances in developing a more rapid procedure based on procainamide gel, it still lacks reproducibility. Therefore, we propose
a. to further elaborate the rapid version of the isolation procedure for HuBuChE.
b. to further work-out the use of an internal standard within this procedure.
c. to explore the use of immobilized pepsin (commercially available) for automation of the procedure.
d. to synthesize a batch of procainamide gel and deliver it to CDC
e. to synthesize a number of phosphylated nonapeptides, to be used as reference standards.

Expected deliverables
- a rapid procedure for isolation and digestion of HuBuChE
- an LC-tandem MS assay for the phosphylated nonapeptide, including the use of an internal standard
- a batch of procainamide gel for isolation of HuBuChE by affinity chromatography.

Ad 3. Generic method for detection of covalent cholinesterase inhibitors
One of the problems with the current assays for assessment of organophosphate exposure is that one has to know in advance for which type of nerve agent to screen for during mass spectrometric analysis. However, it is impossible to screen for adducts of all kinds of inhibitors. Therefore, a generic method for detection of phosphylated HuBuChE is highly needed. In this respect, our attention was raised by the generic method which is used for the detection of phosphorylation sites in proteins in the field of proteomics (Oda et al., 2001). According to this method, proteins are treated with mild base in order to eliminate the phosphate function, resulting in the formation of a dehydroalanine residue in the protein. Subsequently, the thus formed dehydroalanine residue is reacted with a thiol, containing a tag, *e.g.*, a biotin group; the protein is then enzymatically digested and the digest is purified by means of streptavidin affinity chromatography. Recently we have applied this methodology to phosphylated HuBuChE (i.e. after inhibition with a nerve agent) in preliminary experiments. Elimination readily occurred and introduction of various thiol-containing groups proceeded smoothly. Subsequent pepsin digestion gives rise to the formation of the desired nonapeptide. Until yet, we have not found the right “mass tag”, in order to obtain a nonapeptide with an

enhanced response in the mass spectrometer. When this methodology indeed works, "positive" samples can be subjected to further analyses in order to identify the used nerve agent. It is expected that the methodology is suitable for a vast array of organophosphates. In analogy to the proteomics work, we can also apply a biotin-containing tag and isolated the modified nonapeptide by streptavidin affinity chromatography.
We propose to further develop this methodology into an assay that can be applied as a first screening method for large numbers of samples.

Expected deliverable
- a general assay for biomonitoring of exposure to organosphosphates based on the abovementioned technologies.

Ad 4. Development of assays to detect exposure to other toxic chemicals, likely to be used for chemical terrorism

a. Phosgene
Previous research within TNO-PML has shown that upon *in vitro* exposure of human blood to phosgene an intramolecular adduct to Lys-195 and Lys-199 residues in albumin (Noort et al., 2000) is formed. A tandem mass spectrometric assay was developed for analysis of the tryptic fragment containing this intramolecular lysine adduct, which enabled the detection of exposure of human blood to ≥ 1 µM phosgene *in vitro*. This tryptic fragment is a dimer of two peptides, bridged by the carbonyl moiety derived from phosgene. Due to the particular urea bridge in the peptide its fragmentation is restricted to only a few fragments, rendering it very suitable for sensitive LC-tandem MS analysis.
One of the drawbacks of the method is the rather lengthy procedure for albumin isolation and reduction/carboxymethylation of cysteine bridges. In this respect, it is envisaged that the rapid isolation procedure for albumin (see above) can also be applied to this methodology. Furthermore, there have been reports in the literature that describe the on-line reduction/carboxymethylation of proteins (Hsieh et al., 1996; Nadler et al, 1996; Shen et al., 2000), which would render the procedure much less laborious.
In summary, we propose to
- further develop this method for routine use, *e.g.*, by performing the reduction/carboxymethylation of albumin on-line.
- transfer the resulting method to CDC
- synthesize a well-defined amount (0.050 mg) of adducted peptide and transport it to CDC.
  Expected deliverables
- a rapid method for biomonitoring exposure to phosgene, based on adducts to albumin
- tranfer of this method to CDC
- a well-defined amount of reference peptide

The following subjects focus on toxic, readily accessible compounds for which no methods for retrospective detection of exposure are available yet.

b. Cyanide
Cyanide (as HCN or salt) is a readily available toxicant for which no method is available for retrospective detection of exposure. Little is known of any possible adduct formation of cyanide; reaction of cyanide with cystine has been reported to proceed through an intermediate thiocyanate, which subsequently reacts intramolecularly with an amino function

of cystine, giving a five-membered ring. It can be envisaged that a similar reaction might occur with a disulfide function within a protein, followed by further reaction with a nearby lysine residue. We propose to examine this in more detail by exposure of human blood to radioactively labelled cyanide. Covalent attachment of radioactivity to proteins can easily be detected and quantitated. The identity of potential adducts will be elucidated by LC tandem MS and by comparison with synthetic standards. In summary, the feasibility of an assay for monitoring exposure to cyanide, based on adducts to proteins, will be investigated.
Deliverable
- a report describing the proposed research, including full experimental details.

Phosphine
Phosphine is a highly toxic gas which is inter alia used by farmers as a grain fumigant (Newton et al, 1999). It is also an important by-product during the (illegal) manufacturing of metamphetamine (Burgess, 2001). The derivatives aluminum phosphide and zinc phosphide, which generate phosphine upon exposure to moisture, are used as rodenticidal agents (Guale et al, 1994). Especially the phosphide derivatives are regarded by the FBI as potential candidates for use in chemical terrorism because they are readily accessible at low cost.
Whether phosphine can form (persistent) adducts to bio-macromolecules such as proteins is not known. The conversion of oxyhaemoglobin to methaemoglobin has been described in the literature (Chin et al, 1992), but further data have not been reported. We propose whether adduct formation occurs with proteins by using tritiated phosphine, in a similar way as described for cyanide. In case stable adducts with proteins are formed, an assay will be developed for mass spectrometric analysis of the most suitable adduct. In summary, the feasibility of an assay for monitoring exposure to phosphine, based on adducts to proteins, will be investigated.
Deliverable
- a report which includes full experimental details.

II MATERIALS AND INSTRUMENTATION

II.1 Materials

Tripeptide assay

- Human plasma exposed to d0 and/or d8 sulfur mustard (e.g. 1, 5, 10, 50, 100 µM)
- S-HETE-Cys-Pro-Phe for calibration/tuning of instrument
- Pronase, (protease type XIV, Bacterial from Streptomyces Griseus, E.C. 3.4.24.31), Sigma, P 5147
- KH_2PO_4, Fluka,
- KCl, Fluka,
- NH_4HCO_3, Fluka,

Modified Edman degradation

- Human globin from blood that had been exposed to d0 and/or d8 sulfur mustard (e.g. 1, 5, 10, 50, 100 µM)
- Formamide, Fluka, 47671
- Pyridin, 99.9+ %, Sigma Aldrich 270407
- Pentafluorophenyl isothiocyanate PFPITC, Fluka, 76755
- Heptafluorobutyrylimidazole (HFBI), Pierce, 44211
- Sodium chloride, p.a.
- Concentrated hydrochloric acid, p.a.
- Acetonitrile, p.a.
- acetone, p.a.
- diethyl ether, p.a.
- toluene, p.a.
- Dichloromethane, p.a.
- Na_2CO_3, p.a.
- $MgSO_4$, p.a.
- Liquid nitrogen
- Methanol, p.a.
- $KHCO_3$, p.a.
- 2-propanol, p.a.
- heptane, p.a.

Phosgene method

- Reference peptide-phosgene: [ASSAK*QR][LK*ZASLQK], C=O bridge between K* residues, Z= S-carboxymethylcysteine
- Human plasma exposed to various concentrations phosgene
- Trypsin (TPCK treated), Type XIII, E.C. 3.4.21.4, Sigma, T-8642 ca 10.000 units mg/ solid
- Sodium iodoacetate, Fluka, 57858
- Dithiothreitol, Fluka, 43817
- NH_4HCO_3
- Guanidine.HCl
- Tris.HCl
- EDTA

Lewisite method

- Hemolyzed red blood cells isolated from blood exposed to various concentrations of L1
- Heptafluorobutyryl imidazole (HFBI), Pierce, 44211
- 2,3-dimercaptopropanol (BAL), Riedel-de Haen, 64046
- CVAA-BAL
- Phenylarsenic-BAL
- CVAA-BAL-HFB; must be prepared freshly by derivatization of L1-BAL with heptafluorobutyryl imidazole
- Nitrogen or preferably argon.
- Acetonitril, Biosolve
- Toluene, Merck
- Methanol, Merck
- Dichloromethane, Biosolve
- $MgSO_4$, Fluka
- KH_2PO_4, Fluka
- KCl, Fluka
- Bleach

Rapid assay for OP biomonitoring

- Butyrylthiocholine iodide, Sigma (Bornem, Belgium).
- Pepsin (EC3.4.23.1), Roche Applied Science 10108057001 (Almere, the Netherlands)
- 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), Fluka
- Sepharose 4B, Fluka
- Procainamide.HCl, Fluka
- Cyanogen bromide, Fluka
- ε-Aminohexanoic acid and formic acid, Fluka
- NH_4HCO_3
- Na_2CO_3
- NaH_2PO_4
- Na_2HPO_4
- NaCl

Generic assay for OP biomonitoring

- Pepsin (EC 3.4.23.1), Roche Applied Science 10108057001 (Almere, The Netherlands)
- Sepharose 4B, Fluka
- Procainamide.HCl, Fluka
- $Ba(OH)_2$, Riedel-de Haen
- 1H,1H,2H,2H perfluorodecane-1-thiol, Fluorous Technologies Inc. Pittsburgh, PA,
- Ethanethiol, Fluka
- N-Phenylethylenediamin, Fluka
- 4-(2-Aminoethyl)aniline, Fluka
- 2-Phenylethanethiol, Fluka
- 3-(2-Aminoethylamino)propylamine, Fluka
- N-Biotinylated cysteamin, Fluka
- 1,4 Bis-(3-aminopropylamino)butane,
- 2(3-Aminopropyl-amino)ethanol benzylamin (I), Fluka
- 1-(2-Aminoethyl)piperidine, Fluka
- 2-Mercaptoethylamine, Fluka

- 1,3 Bis(3-aminopropyl)1,1,3,3, Fluka
- Tetramethyldisiloxane, Fluka

II.2 Instrumentation/devices

Tripeptide assay

- Plastic tubes, 13 mL, Sarstedt, 60541685
- Centrifuge (4000 rpm), Heraeus Megafuse 1.0 R
- pH meter
- Acrodisc filters, 0.45 µm, PVDF, Waters, WAT200510
- Amicon Ultra-4, centrifugal Filter devices, 10000 MWCO, UFC801096, Millipore
- Centrifuge tubes
- Pasteur pipettes
- disposable syringes, 3.0 cc, slip tip, Aldrich, Z19211-2
- HiTrap Blue HP, Amersham Biosciences, 17-0412-01, 5 x 1 mL columns
- PD-10 columns, Amersham Biosciences, 17-0851-01, 30 pre-packed columns
- 4 mL screw neck vials, 45 x 14.7 mm, Alltech associates, 98110
- 13-425 solid cap. Black propylene with TFE, Alltech, 98430
- LC-MS-MS (triple-quadrupole or Q-TOF instrument)
- Gilson pipettes, 20, 100 and 1000 µL and tips

Modified Edman degradation

- Reactitherm Heating Module 18790, Pierce + Heating block B-1, Pierce, designed for 4 mL vials
- Plastic tubes, 13 mL, Sarstedt, 60541685
- Sample vials clear, 4 mL, Wheaton, 15 x 46 mm, 224802, Aldrich, order no Z18870-0
- Black plastic caps, teflon faced rubber, Wheaton, size 13-425, 240408, Aldrich, order no. Z10642-9
- Vortex Vibrofix, VF1 electronic, Janke & Kunkel
- Vacuum concentrator, Jouan RC10-10
- Centrifuge (4000 rpm), Heraeus Megafuse 1.0 R
- Ultracentrifuge (25000 g), Centrikon T-234
- Gilson pipettes, 20, 100 and 1000 µL and tips
- Measuring cylinders, 100 and 500 mL
- Tubes for ultracentrifuge, resistant to 25000 g or more
- Heat gun
- Vacuum pump, able to reach a vacuum of 0.1 mbar or less
- Glass wool,
- Disposable syringes, 2 mL
- Injection needles
- Tubing, to be used between syringes and pasteur pipettes
- Vials 300 µL, or 200 microliter inserts for larger vials
- Caps for 300 µL vials
- Capper
- Florisil cartridges, classic, Waters, WAT051960
- Pasteur pipettes, long and small
- GC-NICI-MS
- Seppak C18 cartridges, classic, Waters, WAT 051910

Phosgene method

- Reactitherm Heating Module 18790, Pierce + Heating block B-1, Pierce, designed for 4 mL vials
- Plastic tubes, 13 mL, Sarstedt, 60541685
- Sample vials clear, 4 mL, Wheaton, 15 x 46 mm, 224802, ordered from Aldrich, order no Z18870-0
- Black plastic caps, teflon faced rubber, Wheaton, size 13-425, 240408, ordered from
- Aldrich, order no. Z10642-9
- Vortex Vibrofix, VF1 electronic, Janke & Kunkel
- Vacuum concentrator, Jouan RC10-10
- Centrifuge (4000 rpm), Heraeus Megafuse 1.0 R
- Waterbath
- Slide-a-lyzer dialysis cassettes (10 kD, 0.1-0.5 mL, Pierce Prod nr 66415)
- HiTrap Blue Sepharose columns (Amersham Biotech)
- PD-10 columns (Amersham Biotech)
- Pasteur pipettes, long and short
- Gilson pipettes, 20, 100 and 1000 μL and tips
- 10 kD molecular weight cut-off filters (2 mL)
- Glass wool,
- LC/MS/MS (triple-quadrupole or Q-TOF)
- Pepmap C18 HPLC column (LC Packings) or comparable
- Vacuum pump, able to reach a vacuum of 0.1 mbar or less
- Disposable syringes, 2 mL
- Injection needles
- 0.45 micron syringe filters (Waters, 25 mm PVDF, Prod Nr, WAT200510)

Lewisite method

- Seppak C18 cartridges (classic, Millipore)
- Pasteur pipettes, long and small
- Glass wool,
- Plastic tubes, 13 mL, Sarstedt, 60541685
- Sample vials clear, 4 mL, Wheaton, 15 x 46 mm, 224802, ordered from Aldrich, order no Z18870-0
- Black plastic caps, teflon faced rubber, Wheaton, size 13-425, 240408, ordered from
- Aldrich, order no. Z10642-9
- GC/MS, with possibility for EI conditions and a CP-Sil 5 CB column or comparable
- Vacuum concentrator, Jouan RC10-10
- Vacuum pump, able to reach a vacuum of 0.1 mbar or less
- Reactitherm Heating Module 18790, Pierce + Heating block B-1, Pierce, designed for 4 mL vials

Rapid assay for nerve agents

- Amicon (100 kD, 15 mL) and Microcon (3 kD, 0.5 mL) ultra centrifugal filter devices, Millipore (Bedford, MA, USA).
- Peptide synthesis tubes, with filter (2 ml), MultiSynTech, GMBH, Germany
- Heraeus Centrifuge equipped for centrifugation of 100 kD centrifugal devices (Heraeus,Hanau, Germany)

- Eppendorf centrifuge equipped for centrifugation of 3 kD centrifugal devices (Eppendorf. Hamburg, Germany)
- 96 well absorbance reader Asys UVM340 (Eugendorf, Austria)

Generic assay for nerve agents

- Centrifugal ultrafilters (Centricon YM-3, 3 kD or Amicon Ultra-4, 10 kD), were purchased from Millipore, Bedford, MA.
- Further as described for “Rapid assay for nerve agents”

Peptide synthesis

Solid phase peptide synthesis was carried out on a Syro 2000 (Multisyntech, Germany) peptide synthesizer on a 10 µmol scale, using commercially available amino acids and customized Fmoc-based protocols. After synthesis, the peptides were split off from the resin and purified to homogeneity with semi-preparative HPLC.

HPLC

Analytical and semi-preparative HPLC was performed on an AKTA Explorer chromatography system (Amersham Pharmacia Biotech). Analytical column used: Alltech Alltima C18 250 x 4.6 mm; 5 µm particle size. Semi-preparative column used: Alltech Altima C18, 250 x 10 mm; 5 µm particle size

III EXPERIMENTAL PROCEDURES

III.1 Demonstration of procedures

III.1.1 Standard Operating Procedure for albumin – tripeptide assay

The plasma sample (0.5 mL) of interest was spiked with plasma (25-50 µL), isolated from blood exposed to 1 - 100 µM d_8-sulfur mustard. The sample was diluted with buffer A (50 mM KH_2PO_4, pH 7; 2 ml), filtrated over a filter disk (0.45 µm), applied on a HiTrap™ Blue HP cartridge (prepacked with Blue Sepharose High Performance, with Cibacron Blue F3G-A as the ligand; 1 mL) that had been equilibrated with buffer A (10 mL). The cartridge was eluted with buffer A (10 mL) by means of a syringe, within one minute. Subsequently, the cartridge was eluted with buffer B (50 mM KH_2PO_4, 1.5 M KCl, pH 7; 3 mL). The latter effluent was collected. The HiTrap column can be regenerated by washing with buffer B (10 ml) and subsequently with buffer A (10 mL). Next, a PD-10 column (containing 10 mL of Sephadex G 25 material) was equilibrated with 50 mM NH_4HCO_3 (25 mL). The albumin fraction, collected from the HiTrap Blue HP column (3 mL), was applied to the column, and the column was eluted with aqueous NH_4HCO_3 (50 mM; 3 mL). The effluent was collected.

Part of the purified albumin fraction (0.75 ml) was used and subsequently Pronase was added (100 µl of a freshly prepared solution (10 mg/ml) in 50 mM NH_4HCO_3), followed by incubation for 2 h at 37 ºC. The digests were filtrated through molecular weight cut-off filters (10 kD) under centrifugation at 2772 g in order to remove the enzyme. The filtrate was analyzed by means of LC/MS/MS.

For low exposure levels, the filtrate (after 10 kD filtration) was processed on a Seppak C18 cartridge. To this end, the cartridge was conditioned with methanol (5 mL), followed by 0.1% TFA/water (5 mL). The pronase digest was applied, and washed with water (2 mL), containing 0.1 % TFA, 10 % CH_3CN/water (2 mL,0.1 % TFA), 20 % CH_3CN/water (2 mL,0.1 % TFA), and finally eluted with 40% CH_3CN/water (2 mL,0.1 % TFA). The eluate was evaporated to dryness, redissolved in water (0.1 % TFA, 50 microliter and analyzed with LC/MS/MS.

<u>Conditions LC-system</u>
Eluent A: 0.2% formic acid in water. Eluent B: 0.2% formic acid in acetonitrile.

Time (min)	% eluent A	% eluent B	Flow (ml/min)
0	100	0	0.1
5	100	0	0.6
50	30	70	0.6

The flow of 0.6 ml/min was split before the column to 35 µl/min. Column: PepMap C18, 3 µm, 15 cm x 1 mm. Loop: 50 µl.

<u>Conditions triple quad MS</u>
Transitions were monitored of the protonated molecular ions of (S-HETE)Cys-Pro-Phe and (S-d_8-HETE)Cys-Pro-Phe to the most intense fragment (HETE):
MH^+ 470.2 → 105
MH^+ 478.2 → 113
Scan time 1.2 s. Cone voltage 35 V, collision energy 20 eV (Argon pressure 3 x 10^{-3} mBar).

Analyses at TNO (Q-TOF instrument)
LC/electrospray tandem mass spectrometric experiments were conducted on a Q-TOF hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, UK) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a pre-column splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a sixport valve (Valco, Schenkon, Switzerland) with a 10 or 50 µl injection loop mounted and a PepMap C_{18} (LC Packings) or Vydac C18 column (both 15 cm x 300 µm I.D., 3 µm particles). A gradient of eluents A (H_2O with 0.2% (v/v) formic acid) and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation. The flow delivered by the liquid chromatograph was split pre-column to allow a flow of approximately 6 µl/min through the column and into the electrospray MS interface. MS/MS product ion spectra were recorded using a cone voltage between 25 and 40 V and a collision energy between 30 and 35 eV, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar).

Analyses at CDC (triple-quad instrument)
LC-tandem MS experiments during the method demonstration at CDC were recorded on an API4000 triple quadrupole instrument with a standard ionspray interface (Applied Biosystems, Toronto, Canada) and a Shimadzu (Kyoto, Japan) modular liquid chromatograph. In this case the chromatographic hardware incorporated three high pressure pumps, two six-port switching valves, an autosampler with a 50 µL injection loop, and two identical Luna C18 (Phenomenex, Torrance, CA, USA) columns (both 150 mm x 1 mm I.D., 5 µm particles) in parallel. This LC system was configured such that no flow splitting was necessary, and that when the first column was being eluted the second column was being equilibrated in preparation for analysis of the next sample. A gradient of solvents C (H_2O with 1% (v/v) formic acid) and D (80:20 acetonitrile:H_2O plus 1% (v/v) formic acid) was delivered at 50 µL/min. Injections of 50 µL were typically made. The samples were loaded onto the selected analytical column during a 5 min period using Solvent C at 50 µL/min, and then both of the six-port valves were switched. Elution was by means of a linear gradient, ramped from 0% D commencing immediately after the valve switching, to 100% D after a further 25 minutes. During elution of the selected analytical column, Solvent C was used for regeneration of the selected off-line column, at a flow rate of 75 µL/min. The cycle time was 33 minutes. MS/MS (MRM) transitions from *m/z* 470.1 to 105.0 (for the tripeptide) and from *m/z* 478.1 to 113.0 (for the d_8-tripeptide) were recorded at unit resolution on both Q1 and Q3 (i.e., with ion peaks between 0.60 and 0.80 *m/z* units wide at half-maximum height), at a declustering potential of 65 V and a collision energy of 35 eV, with nitrogen as the collision gas.

III.1.2 Standard Operating Procedure for modified Edman degradation

Procedure for modified Edman degradation of globin alkylated by sulfur mustard
Globin (20 mg), from blood exposed to sulfur mustard was mixed with globin (20 mg) isolated from blood exposed to sulfur mustard-d_8 (10 µM) and dissolved in formamide (2 ml). Next, pyridine (8 µl) and pentafluorophenyl isothiocyanate (8 µl) were added and the mixture was incubated at 60 °C in a heating block for 2 h. After cooling to room temperature, the mixture was extracted with toluene (3 × 1 ml) by means of mixing the toluene with the formamide solution using a Vortex (30 s) and centrifuging in a Jouan RC 10.10 centrifugal evaporator for 2 min (1200 rpm). Next, the samples were frozen in liquid nitrogen in order to achieve a better separation of the two layers. The toluene layers were combined, washed with

water (2 × 0.5 ml), aqueous Na_2CO_3 (0.1 M, 0.5 ml) and water (0.5 ml). The organic layer was dried ($MgSO_4$), evaporated to dryness using the centrifugal evaporator and dissolved in toluene (100 µl).
Next, a Florisil cartridge was conditioned with methanol/dichloromethane (1/9, v/v; 2 ml) and dichloromethane (2 ml), respectively. The toluene solution was applied on the cartridge, which was subsequently washed with dichloromethane (2 ml) and methanol/dichloromethane (1/9, v/v; 1 ml). The thiohydantoin was eluted with methanol/dichloromethane (1/9, v/v; 1.5 ml). The latter eluate was evaporated to dryness and dissolved in toluene (100 µl). To this solution heptafluorobutyryl imidazole (10 µl) was added and the mixture was heated at 60 °C for 30 min. After cooling, the reaction mixture was washed with water (2 × 100 µl), aqueous Na_2CO_3 (0.1 M, 100 µl) and finally with water (100 µl). The toluene layer was dried ($MgSO_4$), concentrated to 30 µl and analyzed with GC-MS.

For GC-MS analysis, an HP 5973 mass selective detector was connected to a HP 6890 GC system with an HP 7673 autoinjector, using pulsed splitless injection. The system was operated in the NICI mode (methane) with a source temperature of 150 °C and an ionization energy of 70 eV. The column used was a CPSil 5 CB fused silica capillary column (length 50 m, i.d. 0.32 mm, film thickness 0.25 µm; Chrompack, Middelburg, The Netherlands) or a Hewlett Packard HP-5 column (5% phenyl methyl siloxane; length 30 m, i.d. 0.25 mm, film thickness 0.25 µm). The oven of the chromatograph was kept at 120 °C for 5 min, the temperature was then progammed at 15 °C/min to 275 °C and subsequently kept at this temperature for 10 min. Injection volume was 1 µl (containing about 1% of the total sample).
Ion chromatograms were recorded after monitoring for *m/z* 564 ($M^{-\cdot}$ - 3 HF, analyte) and 572 ($M^{-\cdot}$ - 3 HF, internal standard).

III.1.3 Standard Operating Procedure for phosgene method

Reduction/carboxymethylation of albumin
Guanidine.HCl (M = 95.53, 57.3 g), Tris (hydroxymethyl)aminomethane (M= 121.14, 1.21 g) and EDTA (M= 292.24, 29 mg) were dissolved in water to give a volume close to 98 mL; the pH was adjusted to pH 8.3 with a few drops HCl (6 M) and finally the volume was adjusted to 100 mL with water.
Dissolve DTT (50 mg) in guanidine buffer (3 mL, 6 M guanidine.HCl, 100 mM TRIS, 1 mM EDTA, pH 8.3). Part (0.3 mL) of this solution is added to lyophilized albumin samples that were obtained by means of affinity chromatography, as described for the tripeptide assay. These solutions are incubated at 55 ºC for 40 min. Next, iodoacetic acid (10 mg) is added and the mixture is incubated for 30 min at 40 C , followed by dialysis against 50 mM NH_4HCO_3 (3x 1L) at RT in a Slide-A-Lyzer dialysis cassette (10000 MWCO, 0.1-0.5 mL). The last dialysis step is performed overnight at RT.

Trypsin digestion
Dialyzed (reduced and carboxymethylated) albumin was transferred from the slide-a-lyzer cassettes into vials (4 mL). Next, trypsin was added (2 % w/w). For this purpose, trypsin (6 mg) was dissolved in buffer (5 mL, 50 mM NH_4HCO_3). Part (50 microliter) was added to the dialyzed solution of reduced and carboxymethylated albumin in buffer. Incubation was performed at 37°C for 4 h. Finally, the sample was filtrated through a pre-rinsed (50 mM NH_4HCO_3, 3x 1 mL) filter with a 10 kDa cutoff with centrifugation at 3200g, to remove the

enzyme. The resulting trypsin digests were analyzed by means of LC tandem mass spectrometry.

ESI-LC-tandem MS conditions:
Eluent A: 0.2 % formic acid/water
Eluent B: 0.2 % formic acid acetonitril
A gradient was applied from:
100 % A (5 min, 0.1 mL/min)
100 to 30 %A in 60 min (0.6 mL/min)
The flow of 0.6 mL/min was reduced by a LC-packings splitter to ca. 40 microliter/min.
Column: PepMap C18, 3 micron, 15 cmx 1 mm, loop: 10 microliter
MS conditions (Q-TOF):
Full scan product ion spectra (MS/MS) were recorded of the doubly charged native adduct peptide (MH_2^{2+} 861.2) and of the [^{13}C] analogon (MH_2^{2+} 861.7) at a cone voltage of 35 V and a collision energy of 35 eV. Argon gas pressure was 10^{-4} mBar.
For more sensitive detection of (ASSAK*QR)(LK*CcmASLQK), analyses were performed in the multiple-reaction monitoring (MRM) mode (transitions $[M + 2H]^{2+}$ *m/z* 861.0 >747.5 and $[M + 2H]^{2+}$ m/z 861>773.5 on a Quattro II triple-quadrupole mass spectrometer. Operating conditions were as follows: cone voltage 40 V; collision energy 30 eV, argon pressure, 5 x 10^{-3} mBar; and dwell time, 1 s. LC conditions were as described above.

III.1.4 Standard Operating Procedure for Lewisite method

Reagent preparation:
BAL (M = 124.23, d = 1.239 g/mL, 1 mL = 10 mmol) solution: dilute 10 microliter BAL (100 micromole) with 990 microliter acetonitril to achieve an end concentration of 100 mM.

Internal standard preparation:
PAB (M=274) solution: dissolve 1 mg to an end concentration of 100 micromolar in acetonitril; 1 mg=3.64 micromole. In 3.64 mL: 1 mM. Dilute this solution 1:10 to give 100 micromolar.

Sample work-up
Incubate lysed erythrocytes (1 mL) with BAL solution (10 microliter) and PAB solution (100 micromolar , 10 microliter) for 1 h at RT.
Add water (9 mL) to the lysed erythrocytes, apply to a conditioned Seppak C18 classic cartridge (conditioned by washing with methanol (10 mL), water (10 mL) consecutively), and wash with water (20 mL). Elute L1-BAL and PAB with dichloromethane/acetonitril (4/1, v/v, 3 mL), concentrate with a vacuum concentrator after a first volume reduction using a stream of air , coevaporate (2 x 0.5 mL toluene), dissolve in toluene (100 microliter) and add HFBI (10 microliter). Heat (50 ºC) for 1 h. Next, wash with water (4 x 100 microliter). Dry over $MgSO_4$ and analyze with GC/MS.

GC-MS conditions
Mass spectrometer (Agilent 5973N MSD)
Vacuum: 10^{-6} Torr
Source temperature: 200 ºC
Multiplier: 2200 V
Emission: 34.6 microampere

ionisation energy: 70 eV
Ionisation mode: EI
Acquisition mode:SIM
Dwell time: 100 ms
Mass: m/z 454 and 470
Type recording: GC-MSD
Injection volume: 1 microliter, splitless

Gas chromatograph (HP 6890)
Column: factorfour VF-5ms, 50 m x 0.25 mm, df 0.25 micrometer
Carrier gas: helium, constant flow, 1 mL/min
Temperature: 120 (1), 8 ºC/min, 280 (5)
Interface: 280 ºC

III.2 Rapid assay for OP biomonitoring

III.2.1 Preparation of procainamide gel

Approximately 125 mL of Sepharose 4B gel suspension in ethanol/water was placed in a funnel with a glass filter and washed with 1 liter of water, re-suspended in 0.2 M phosphate buffer (pH 11.5; 150 ml) and cooled at 4 °C. Cyanogen bromide (7 g in 15 mL acetonitrile/water 1:1) was added gently and the suspension was stirred for 10 min. Next the suspension was washed with water (1 L) and immediately transferred into ε-aminohexanoic acid solution (1.6 g ε-aminohexanoic acid in 150 mL 0.2 M sodium carbonate, 0.2 M sodium bicarbonate and 0.4 M sodium chloride, pH 9). The mixture was stirred for 48 h at 4 °C. Next the gel was washed with water, re-suspended in water (150 mL) and after addition of 4.2 g procainamide.HCl adjusted to pH 4.5. EDC (N-3-dimethylaminopropyl)-N-ethylcarbodiimide; 7 g) was added and the pH adjusted again to pH 4.5. The pH slowly rose, so the pH had to be adjusted a few more times. When the pH remained relatively stable, the mixture was stirred in the cold room for 48 - 60 h. Next, the gel was washed with water (1.5 L). The wash fluid was collected and extinction was measured at 278 nm ε^{270} =16150 $M^{-1}cm^{-1}$). Calculation revealed that 29 µmol of procainamide was bound to 1 mL of gel.
The gel is stored in water (150 ml), containing 0.02% NaN_3.

III.2.2 Inhibition of plasma samples with nerve agent

Human plasma was inhibited with sarin, d7-sarin, soman, VX or tabun. The concentration of the nerve agent in plasma was 1 µg/ml which corresponds with 3.7 – 7 µM which is a 75- 140 fold excess compared to the concentration of BuChE (50 nM). Inhibition of the sample was allowed for 2 hrs at room temperature. Next 0.5 ml of nerve agent inhibited plasma was mixed with 0.5 ml d7-sarin inhibited plasma (internal standard). For one sample (blank) 0.5 ml non-inhibited plasma was mixed with 0.5 ml d7-sarin inhibited plasma. The plasma samples were further processed as described below.

III.2.3 Isolation of HuBuChE from human plasma

A disposable 10 mL mini-extraction column (tube AIMED AMS 422 peptide synthesizer, Gilson, Villiers le Bel, France) was filled with 2 mL procainamide-gel, which was washed with 20 mL of phosphate buffer (15mM NaH_2PO_4 and 5 mM Na_2HPO_4, pH 6.9) Then, 1 mL

of plasma sample was gently mixed with the procainamide-gel. After 30 min at room temperature, the gel was washed with 5 mL phosphate buffer and 5 mL 150 mM sodium chloride (150 mM NaCl in phosphate buffer). Finally, HuBuChE was eluted with 7 ml 600 mM NaCl in phosphate buffer.

III.2.4 Digestion of HuBuChE with pepsin

BuChE solution obtained after procainamide affinity extraction was concentrated using a 100 kD cut-off filter. The retentate was washed with 5% formic acid (2 x 2 mL). The retentate (approximately 200 µL) was transferred to a 4 ml glass vial; the filter was rinsed with 250 µl 5% formic acid. The rinse fluid was combined with the retentate. Pepsin solution (50 µl of a 0.2% (i.e., 2 mg/ml) solution in 5% formic acid) was added. After incubation for 2 h at 37 °C, the incubation mixture was filtrated through a pre-washed (0.5 ml water) 3 kD cut-off filter. The filter was washed with 150 µl 5% formic acid solution and the fluid was filtrated and pooled with the first filtrate. In case of 100% inhibition, this solution was used for LC-tandem MS experiments. In case a low observable detection level was desired, the combined filtrates were concentrated and the residue was dissolved in water with 5% formic acid (100 µl) and analyzed with LC-tandem MS.

III.2.5 LC-tandem MS of pepsin digests

The LC system consisted of an Alliance 2690 HPLC gradient system (Waters, Milford, MA, USA). The mass spectrometer was a Q-TOF instrument (MicroMass, Altrincham, UK) equipped with a standard Z-spray electrospray interface. Stationary phase was a PepMap C18 column (15 cm x 1000 µm, 3 µm particles) from LC-Packings (Amsterdam, The Netherlands). The mobile phase consisted of a gradient of A: 0.2% formic acid in water and B: 0.2% formic acid in acetonitrile. Gradient program was 0'-5': 100%A, flow 0.1-0.6 mL/min; 5'-60': 100% A->70% A, flow 0.6 mL/min. The pump flow (0.6 mL/min) was reduced to a column flow of 40 µL/min by a splitter (LC-packings). Injection volume was 10 µl. Electrospray MS-MS spectra of the protonated molecular ion were recorded using a cone voltage of approximately 35 V and collision energy of approximately 30 eV. Subsequently, ion chromatograms of m/z 778.4 that were obtained after selection and fragmentation of the protonated molecular ion, were recorded.

III.2.6 Ellman assay

The wells of a 96 well plate were filled with 100 µl DTNB solution (0.8 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 100 mM phosphate buffer, pH 8.0). Next 10 µl of the sample was added wherafter 100 µl BuSChI solution (0.8 mM Butyrylthiocholine iodide in water) was added. The plate was stirred for 10 s and the absorbance at 412 nm was read in the well plate reader. After a certain period the absorbance was measured again. The net increase of the absorbance in time is a measure of the HuBuChE activity in the sample.

III.3 Generic assay for OP biomonitoring

III.3.1 Alkaline hydrolysis of inhibited HuBuChE followed by Michael addition of ethanethiol and pepsin digestion

Purified HuBuChE (250 microliter of a stock solution; 5 nmol) was 100 % inhibited with either VX, sarin or soman (10 equivalents). The solution was concentrated over a 10 kD ultrafilter (4000 rpm, 30 min) to remove excess inhibitor and the retentate was dissolved in an aqueous solution of $Ba(OH)_2$ and ethanethiol (100 and 50 mM, respectively; 0.5 mL). The mixture was incubated for 2 hours at 37 ºC and neutralized with TFA/CH_3CN (1 %). The samples were concentrated over a 10 kD filter and washed with a solution of NH_4HCO_3 (50 mM, 3 x 1 mL). After the third wash step, the concentrate was dissolved in aqueous formic acid (5 %, 0.55 mL) containing pepsin (0.10 mg). The mixture was incubated for 2 h at 37 ºC, after which the mixture was concentrated over a 3 kD ultrafilter. The filtrate was analyzed with LC/MS/MS.

III.3.2 Michael addition of ethanethiol after pepsin digestion of (inhibited) HuBuChE

Purified HuBuChE (blank or inhibited; 1 nmol) was digested with pepsin as described above. After filtration of the peptic digest over a 3 kD filter, the filtrate was now lyophilized to dryness and redissolved in 200 microliter of $Ba(OH)_2$ (100 mM) and ethanethiol (50 mM). After 1 h at 37 ºC, the reaction was quenched with acetic acid (5 microliter) and the mixture was used for analysis with LC/MS/MS.

III.3.3 Generic procedure for diagnosis of exposure to nerve agents

Isolation of HuBuChE from plasma and subsequent pepsin digestion was carried out as described previously. The filtrate was concentrated, coevaporated with 50 mM NH_4HCO_3 (2x 0.5 ml), and dissolved in an aqueous solution of $Ba(OH)_2$ and 2-(3-aminopropylamino)ethanol (100 and 50 mM, respectively; 0.2 mL). After incubation for 1 h at 37 ºC, the reaction was quenched by the addition of acetic acid (10 microliter). The resulting solution was analyzed with LC/MS/MS.

MS conditions used:

Eluent A: H_2O(0.2 % formic acid)

Eluent B: CH_3CN (0.2 % formic acid)

Gradient:	Time	%A	%B	Flow (mL/min)
	0	100	0	0.1
	5	100	0	0.6
	60	30	70	0.6

The flow of 0.6 mL/min was reduced by a LC-packings splitter to a column flow of ca 40 microliter/min.

Column: PepMap C18, 3 µm, 15 cm*1 mm, loopsize 10 microliter

Optimization MS/MS (parent ion, collision energy).

Parent ion	Fragment ion (peakintensity eV)	Fragment ion (peakintensity eV)
MH_2^{2+} 448.7,	720.3 (429 at 18 eV)	791.4 (583 at 18 eV)

III.4 Synthesis of reference materials

III.4.1 (S-HETE)Cys-Pro-Phe for albumin tripeptide assay

Coupling

First, immobilized PF (120 µmol) was prepared on a Syro peptide synthesizer according to standard protocols. Subsequently, coupling with Fmoc-Cys-S-HETE-OtBu was effected on a 90 µmol scale to give S-(2-Hydroxyethylthioethyl)-CPF.

Briefly, the resin was swollen in dichloromethane (5 ml) for 30 minutes and the peptide coupling reagents mixture was prepared. PyBop (281 mg), HOBt (73 mg) and Fmoc-Cys-HETE-OtBu (232 mg) were dissolved in N-methyl-2-pyrrolidone (NMP; 700 µl).

The coupling reaction was performed in two separate columns A (50 µmol) and B (40 µmol), due to the relatively high volumes. To column A the reagents mixture (388 µl), diisopropylethylamine (105 µl) and extra NMP (100 µl) were added. Column B was also treated with the mixture (312 µl), diisopropylethylamine (84 µl) and NMP (100 µl). The reaction mixtures were kept under argon for two hours.

Fmoc removal

The resin was washed with NMP (5 x 3 ml), treated with 20% piperidine/N,N-dimethylacetamide (DMA) (3 x 1 ml) for 10 minutes and washed with DMA (3 x 3 ml). Finally the resin was washed with NMP (5 x 3 min).

Cleavage

The synthesized S-HETE-CPF was cleaved from the resin with trifluoracetic acid/ triisopropylsilane (95/5) according to a standard protocol, precipitated with diethyl ether/pentane (1/1), washed and dried. The peptide was taken up into MQ/acetonitril and analysed with HPLC.

Purification and quantification

The product ((S-HETE)Cys-Pro-Phe) was purified with HPLC, analysed with LC-MS and concentrated under vacuo. The peptide (weighed amount: 11 mg) was dissolved in a mixture of MQ and acetonitrile (1/1, 2 ml) and quantified with HPLC by comparison with an already quantified standard (total yield: 10.05 mg). Finally, the peptide solution was divided into small portions (50 µg, 250 µg and 1 mg) and concentrated.

ES-MS data: *m/z* 470 (MH^+); 453 (MH^+ - NH_3); 364 MH^+ - $H_3CCH_2SCH_2CH_2OH$); 263, y_2''; 166, y_1''; 137, $^+SCH_2CH_2SCH_2CH_2OH$; 105, $^+CH_2CH_2SCH_2CH_2OH$.

III.4.2 Synthesis of pentafluorophenylthiohydantoin derivative of N-terminal valine for modified Edman degradation of sulfur mustard modified hemoglobin

First, a $KHCO_3$/2-propanol solution was prepared by dissolving 0.5 M $KHCO_3$ (100 ml) in 2-propanol (50 ml). N-(2-Hydroxyethylthioethyl)valine (50 mg ; 0.21 mmol) was dissolved in $KHCO_3$/2-propanol (20 ml ; pH = 8.84) and pentafluorophenylisothiocyanate (25 µl ; 0.8 eq.) was added. The mixture was stirred and heated in a water bath (40^o C) for 2 hours. Subsequently, the mixture was cooled to room temperature and extracted with hexane (2 x 25 ml). The hexane layer was concentrated under reduced pressure until a colorless sirup remained. The residue was taken up into dichloromethane (200 µl) and purified using a short column of silica gel (1% methanol/dichloromethane as eluent). The fractions were analysed by TLC (1% methanol/dichloromethane) and GC-MS: in each case one spot/peak could be

observed. The purified product was dissolved in $CDCl_3$ and quantified: 56 mg. The solution was concentrated under vacuo and the residue was taken up in dichloromethane (1 ml), divided into small portions (1 and 10 mg), concentrated and stored at -70 ºC.
GC-MS (EI) data: *m/z*, 60 $[C_2H_4]^{+\cdot}$, 104 $[CH_2CHSCH_2CH_2OH]^{+\cdot}$, 130 $[C_6H_{12}NS]^+$, 351, loss of $^{\cdot}SCH_2CH_2OH$, 383, loss of $^{\cdot}CH_2CH_2OH$, 410, loss of H_2O, 428, $M^{+\cdot}$.

III.4.3 Synthesis of phosgene albumin adduct O=C-(T25-28)

N-Fmoc-cysteine-(S-carboxymethyl tert-butyl ester)
Cysteine.HCl (10.3 mmol; 1.63 g) was dissolved in 50 mM NH_4CO_3 solution (100 ml). The pH of the reaction mixture was brought to 8.2 with NH_4HCO_3. Acetonitrile (50 ml) and t-butyl bromoacetate **(**2.4 g; 1.8 ml; 12.2 mmol) were added; the pH of the reaction mixture slowly decreased to 8.0. After stirring for 2 h at room temperature, TLC analysis (eluent: ethylacetate/n-butanol/acetic acid/water, 2/1/1/1, v/v/v/v; colorization with I_2) showed complete conversion into a higher running product. The reaction mixture was concentrated and subsequently the concentrate dissolved in water/dioxane (2/1, v/v, ca. 150 ml). The pH of the solution was brought to 8 with Na_2CO_3. Fmoc-Cl (13.5 mmol; 3.5 g), dissolved in 20 ml acetone, was added in portions. After stirring for 1 h, TLC analysis (eluent 8% $MeOH/CH_2Cl_2$) showed complete conversion. After bringing the pH to 8 with aqueous NaOH, the misture was extracted with petroleum ether. Subsequently, the mixture was acidified to pH 3.5 and extracted with ethyl acetate. The ethyl acetate fraction was dried ($MgSO_4$) and concentrated. Yield: 1.5 g (33% for two steps). Electrospray LC-MS: m/z 480 (MNa^+), 458 (MH^+), 402 ($MH^+ - C_4H_8$).

O=C-(T25-T28)
Peptide synthesis was performed according to standard protocols for solid phase peptide synthesis.
The peptides (Z-Ala)-Ser-Ser-Ala-Lys-Gln-Arg(NO_2) (4.9 mg; 5.3 µmol) and (Z-Leu)-Lys-Cys(S-carboxymethyl)-Ala-Ser-Leu-Gln-Lys(Z) (6.2 mg; 5.1 µmol) were dissolved in water/acetonitrile (v/v, 1/1; 5 ml). The pH of the solution was brought to 9 with diluted NaOH solution. A 20% solution of phosgene in toluene (0.1 ml) was diluted with acetonitrile (0.9 ml). Small aliquots (25 µl) of this solution were added to the peptides solution, after which the pH of the solution was brought to pH 9 with aqueous NaOH (40 mM). It was verified with LC tandem MS that, in addition to the symmetrical products, the desired product had been formed. The desired product was isolated by means of semi-preparative reversed phase HPLC. Yield: 1.2 mg.
A part (0.6 mg) of the protected product was deblocked by treatment with HF in anisole in a Teflon container. To the peptide anisole (50 µl) and liquid HF (1 ml) was added. The reaction mixture was stored for 2 h at 0 ºCt. HF was removed under a stream of N_2 gas; the residue was dissolved in 0.2 M acetic acid (2 mL). The solution was extracted with diethylether (2 ml). Purification of the crude product was effected by means of reversed phase HPLC (Source kolom). Yield: 175 µg.

III.4.4 Synthesis of standards for Lewisite method

Synthesis of CVAA-BAL

To a solution of L1 (415 mg; 2 mmol) in acetone (30 mL), BAL (286 mg; 2.3 mmol) was added in small amounts within 30 min. Subsequently, the mixture was concentrated, and CVAA-BAL was isolated and purified using silica gel column chromatography, with toluene/ethylacetate (100/0 to 95/5, v/v) as the eluent. Appropriate fractions (analyzed using TLC; eluent: ethyl acetate/toluene, 1/4 ,v/v) were collected and concentrated. Yield: 76 mg (0.3 mmol, 15%) of a slightly colored oil. The product was a mixture of two isomers (ratio: 7/3) as determined by NMR spectroscopy. CVAA-BAL (code: 0439AF01; 5 vials containing 1 mg each). The samples were stored at CDC at -70 ºC.

^{1}H-NMR ($CDCl_3$):

δ (ppm): 6.65 (d, 1H,J= 14.3 Hz, = C(Cl)H, isomer II), 6.55 (d, 1H, J=14.3 Hz, =C(As)H, isomer II), 6.52 (m, 2H, J= 14.2 Hz, CH =CH, isomer I), 4.14-3.10 (m, 5H, 2 x CH_2, 1 x CH; both isomers), 1.96 (b, 1H,OH, isomer I), 1.88 (b, 1H, OH, isomer II).

^{13}C-NMR ($CDCl_3$):

δ (ppm): 136.5 (=C, isomer II), 134.7 (=C, isomer I), 128.0 (=C, isomer II), 127.7 (=C, isomer I), 64.5 (CH_2OH, isomer I), 63.7 (CH_2OH, isomer II), 62.5 (CH, isomer II), 58.7 (CH, isomer I), 44.0 (CH_2, isomer II), 43.2 (CH_2, isomer I).

Synthesis of internal standard phenylarsine-BAL

Phenylarsine-BAL (PAB), which was used as an internal standard, was prepared starting from phenylarsine oxide and purified as described for CVAA-BAL. Yield: 630 mg (70%). The product was a mixture of two isomers (ratio 7/3), according to ^{1}H-NMR. Phenylarsine-BAL (code 0441AF02; 5 vials containing 1 mg each).

The samples were stored at CDC at -70 ºC.

^{1}H-NMR ($CDCl_3$):

δ (ppm): 7.4-8.0 (m, 5H, H-aromatic), 5.2 (m, 1H, OH), 4.0-2.8 (m, 5H, 2 x CH_2, 1 x CH)

^{13}C-NMR ($CDCl_3$):

δ (ppm): 129-128 (C-aromatic), 63.3 (CH_2OH; isomer I), 63.0 (CH_2OH, isomer II), 62.4 (CH, isomer I), 58.4 (CH, isomer II), 43.1 (CH_2, isomer II), 42.3 (CH_2, isomer I).

III.4.5 Synthesis of reference peptides for rapid assay for nerve agents

Synthesis of bis(di-isopropylamino)chlorophosphine

To a solution of phosphotrichloride (14ml, 0.158 mol) in diethyl ether (240 ml) di-isopropylamine (95.8g, 0.947 mol) was slowly added under argon atmosphere. The mixture was stirred for 6.5 hours at reflux temperature and 16 hours at room temperature. The di-isopropylamine•HCl salt was removed by filtration and the solvent by evaporation. The residue was distilled under atmospheric pressure in order to remove the remaining di-isopropylamine, followed by distillation under reduced pressure (4 mmHg, 140ºC). The product distilled at a temperature of 115 – 118 ºC.

Yield 34.4 g (82%); ^{31}P NMR (85% H_3PO_4) δ (ppm): 133.5.

Synthesis of bis(di-isopropylamino)methylphosphine

To a cold (acetone/CO_2 bath) solution of bis(di-isopropylamino)chlorophosphine (13.29g, 49.8mmol) in diethyl ether was added dropwise a solution of methylmagnesiumbromide (17ml, 51.0mmol, 3.0M in diethyl ether) under an argon atmosphere. After 30 minutes at low temperature the reaction mixture was stirred for 1 hour at room temperature. The precipitate was removed by filtration and the filtrate was distilled under normal pressure in order to remove the solvent. This was followed by distillation under reduced pressure (1mbar, 110ºC).

The product distilled at a temperature of 92-93 ºC. Yield: 9.43 g (77%). ^{31}P NMR (85% H_3PO_4) δ (ppm): 39.9.

Synthesis of O-isopropyl-bis(di-isopropyl)phosphoamidite (A)
Isopropyl alcohol (536µl, 421mg, 7.0 mmol) and sym-collidine•HCl (80 mg, 0.5mmol), that had been dried by coevaporation with acetonitrile (3 x 5ml), were dissolved in dichloromethane (5ml). Bis(diisopropylamino)methylphosphine (1.24 g, 5 mmol) was added and the solution was stirred for 24 hours at room temperature. TEA (0.5ml) was added and the reaction mixture was concentrated. The product was purified by silica gel column chromatography with hexane/TEA (19/1, v/v) as eluent. Yield: 0.90 g (88%).

Synthesis of O-ethyl bis(di-isopropyl)methyl phosphoamidite (B)
This reagent was prepared as described for the synthesis of reagent A; ethanol (410 µl, 323mg, 7.0mmol) was used instead of isopropyl alcohol. Yield: 0.78 g (82%).

Synthesis of O-p-methoxybenzyl bis(di-isopropyl)-methylphosphoamidite (C)
This reagent was prepared as described for the synthesis of reagent A; methoxybenzyl alcohol (870 µl, 962mg, 7.0mmol) was used instead of isopropyl alcohol. Yield 1.15 g (82%).
^{31}P NMR (85% H_3PO_4) δ (ppm): 122.2.

Synthesis of bis(dimethylamino)ethoxyphosphine
To a solution of ethyldichlorophosphite (5.5 g; 37.4 mmol) in diethylether (20 ml) was added a solution of dimethylamine (6.11 g, 135.8 mmol) in diethylether (20 ml), while the mixture was kept under an Argon overpressure at 10 ºC. The reaction mixture was stirred for 3 h. The formed dimethylamine.HCl salt was removed by filtration and the filtrate was concentrated. The residue was distilled under reduced pressure (12-14 mm Hg) at 60 ºC. The product distilled at a temperature of 45 ºC.
^{31}P NMR (85% H_3PO_4) δ (ppm): 137.7.

Synthesis of O-p-methoxybenzyl(N,N-dimethyl)ethoxyphosphoroamidite (D)
This reagent was prepared as described for the synthesis of reagent A. In this case bis(dimethylamino)ethoxyphosphine (825 mg, 5 mmol) was used instead of bis(diisopropylamino)methylphosphine, and methoxybenzyl alcohol (962 mg, 7.0 mmol) was used instead of isopropanol. Yield: 0.464 g (30%). ^{31}P-NMR (85% H_3PO_4) δ (ppm): 146.4.

Phosphorylation of the immobilized nonapeptide
First, the protected and immobilized nonapeptide Boc-FGE(tBu)SAGAAS(tBu) (10µmol) was washed consecutively with dimethylacetamide, t-amyl alcohol, 20% acetic acid in t-amyl alcohol, t-amyl alcohol, diethyl ether and under vacuum dried on P_2O_5. Phosphitylating reagent (0.15mmol, 15eq in 800µl acetonitril) and ^{1}H-tetrazole (167µl, 0.075mmol, 0.45M in acetonitril) were added to the immobilized peptide and the mixture was stirred for 16 hours. *tert*-Butyl hydroperoxide solution (80% in water; 100 µl) was added and the mixture was stirred for one hour. The peptide (still immobilized) was once more washed according to the previous described method.
In case of isopropyl methylphosphonyl nonapeptide, reagent A was used, in case of ethyl methylphosphonyl nonapeptide reagent B, in case of methylphosphonyl nonapeptide reagent C and in case of O-ethylphosphoryl nonapeptide reagent D.

Cleavage from the resin and deprotection
The immobilized phosphonylated peptide was cleaved from the resin and deprotected by treatment with a TFA/TIS solution (95/5, v/v); 200 µl of this solution was added six times every 5 minutes. After the last addition, the resin was filtered off and left in the TFA/TIS solution for 2 hours after which the solution was removed. After dissolving the residue in MQ followed by freeze drying the desired peptide adduct was obtained. They were purified by semi-preparative HPLC using an Altima C18 column.

Analytical data (TOF MS ES^+):
"nonapeptide" denotes FGES*AGAAS, with S* phosphylated.
Isopropyl methylphosphonyl nonapeptide: m/z 916.4 (MH^+), 458.7 (MH_2^{2+})
Ethyl methylphosphonyl nonapeptide: m/z 902.3 (MH^+), 451.7 (MH_2^{2+})
Methylphosphonyl nonapeptide: m/z 874.3 (MH^+), 437.7 (MH_2^{2+})
Ethyl phosphoryl nonapeptide: m/z 904.3 (MH^+), 452.7 (MH_2^{2+})

Analytical data (TOF MS/MS ES^+):
Characteristic, unique fragments:
Isopropyl methylphosphonyl nonapeptide: no MS/MS available of synthetic peptide adduct.
Ethyl methylphosphonyl nonapeptide: m/z 442.7(MH_2^{2+} - H_2O).
Methylphosphonyl nonapeptide: m/z 428.7 (MH_2^{2+} - H_2O).
Ethyl phosphoryl nonapeptide: m/z 443.7 (MH_2^{2+} - H_2O).

All peptides seem to loose the phosphonyl moiety easily during fragmentation. Characteristic common fragments:
778.3 (MH^+ - phosphonyl moiety).
673.3 (b_8 – phosphonyl moiety).
602.3 (b_7 – phosphonyl moiety).
531.2 (b_6 – phosphonyl moiety).
177.1 (a_2/y_2'').
106.1 (y_1").

Annex to experimental part; detailed reports of methods transfers

1. Demonstration of albumin tripeptide assay

This assay was demonstrated simultaneously to US Army and to CDC; see previous annual report.

2. Modified Edman degradation

(Monday, November 8, 2004)

Standards

Reference standard 9705AF03 (pentafluorophenylthiohydantoin of valine-sulfur mustard adduct; 1.8 mg according to quantitative ^{1}H-NMR analysis), was dissolved in CH_3CN (180 µl), to give a 10 mg/ml stock solution. 3 further dilutions were prepared: 0.1 mg/ml, 1 µg/ml, 10 ng/ml (no codes were given).

The 1 µg/ml solution was used for setting-up the LC tandem MS method. Various ion combinations for MRM were explored; strongest intensity was obtained for *m/z* 429 → *m/z* 130 (identity of this fragment is still unknown).

The HR-GCMS Mat 900 was further optimized with already processed samples 0440AF01-05 for analysis under EI conditions (this is the most sensitive mode on this instrument).

Processing of globin samples

Globin samples were weighed: see Table 1.

Table 1. Globin samples processed by modified Edman degradation

Globin sample	Description	Amount used (mg)	Internal standard	Sample code after modified Edman degradation
0442AF01	blank	24.4	0442AF07; 50 µl	0446AF01
0442AF03	10 nM	24.1	0442AF07; 50 µl	0446AF02
0442AF04	100 nM	20.0	0442AF07; 50 µl	0446AF03
0442AF05	1 µM	21.2	0442AF08; 50 µl	0446AF04
0442AF06	10 µM	22.2	0442AF08; 50 µl	0446AF05
0442AF07	d_8, 2 µM	25.1 in 1.25 ml formamide	n.a.	n.a.
0442AF08	d_8, 100 µM	28.7 in 1.43 ml formamide	n.a.	n.a.
0442AF09	d_0/d_8, 5/5 µM	23.9	n.a.	0446AF06

The weighed globin samples were dissolved in the appropriate amounts of formamide according to the work-plan. Incubation with pentafluorophenyl isothiocyanate was effected before 12 pm (2 h reaction time, during lunch).

Work-up of the modified Edman degradation reactions according to the procedure described in the work-plan. After 3x extraction with toluene, the combined toluene layers were washed consecutively with water (2x 0.5 ml), aqueous 0.1 M Na_2CO_3 (0.5 ml), water (0.5 ml), and dried on $MgSO_4$. The samples were evaporated to dryness with a Zymark TurboVap Evaporator under heating at 30 ºC by applying a N_2 flow, dissolved in toluene (200 µl; normally 100 µl when samples are not split) and stored as such at –20 ºC. The toluene-layers of the first run of modified Edman degradations were split in two equal portions: one portion for the LC-MS method and 1 portion for the GC-MS method (after derivatization with heptafluorobutyryl imidazole). The samples for the LC-tandem MS method were evaporated to dryness and subsequently dissolved in CH_3CN/H_20, 20/80, v/v (100 µl).

Codes of the various samples (underivatized!!): see Table 1.

Codes for the LC-tandem MS samples (after splitting): see Table 2.

Table 2. Samples for LC-tandem MS analysis

Sample code	description
0446AF07	Half of 0446AF01; blank/d_8
0446AF08	Half of 0446AF02; 10 nM/d_8
0446AF09	Half of 0446AF03; 100 nM/d_8
0446AF10	Half of 0446AF04; 1 µM/d_8
0446AF11	Half of 0446AF05; 10 µM/d_8
0446AF12	Half of 0446AF06; 5 µM/5 µM d_8

LC tandem MS analysis of samples 0446AF07 and 0446AF12 was performed overnight.

(Tuesday, November 9, 2004)

LC tandem MS analysis

Preliminary results of LC-tandem MS analyses of samples 0446AF07 and 0446AF12 were slightly disappointing: relatively small signals were obtained for the analyte. It was decided to first focus on the 0446AF12 sample, and then to continue with the other samples 0446AF08-0446AF11 (see Table 2). The 0446AF08-11 samples were run overnight (Tuesday → Wednesday).

Analysis of blood samples of accidentally exposed person

From blood sample labelled 04-905-0035-SPRB2 (under top label the sample was labelled 04-905-0035-P1; under the latter label was the original label "Mould 081704"; hemolyzed blood) and blood sample labelled 04-905-0031-SPRB2 (under top label the samples were labelled 04-905-0031-B1; under the latter label was the original label "Mould 072204"; whole blood), 0.5 ml (half of total sample) was pipetted into Eppendorf tubes. Codes for the two portions of samples that will be processed: 0446DN01 (Mould 081704) and 0446DN02 (Mould 072204).

Centrifuged (in Eppendorf tubes) in order to see whether there was still any layer separation. No layer separation could be observed. To each sample water (0.5 ml) was added in order to allow for (further) hemolysis (30 min). Subsequently, globin was isolated according to the commonly used procedure by precipitation in HCl/acetone, followed by washing with acetone and diethyl ether. The entire procedure was performed in the presence of a CDC scientist. The obtained globin material appeared similar to globin samples obtained after isolation of regular blood samples. The globin samples were air-dried, after which they will be subjected to modified Edman degradation.

Codes: 0446DN03 (120 mg) is derived from 0446DN01. 0446DN04 (93 mg) is derived from 0446DN02.

GC-EI-MS analysis of already processed samples 0440AF01-05; HFB derivatization of standard.

Analysis did not proceed quite satisfactory yet. It was decided to derivatize a small amount of 9705AF03 with heptafluorobutyrylimidazole, for use as reference for the GC-MS analysis. Thus, 10 µl of the 10 mg/ml stock solution of 9705AF03 in CH_3CN was diluted to 1 ml with toluene. Subsequently, heptafluorobutyric imidazole (100 µl) was added and the reaction mixture was incubated at 60 ºC for 30 min. After washing, drying etc. a clear solution resulted

(0446AF13; approximately 0.1 mg/ml). Prior to analysis, this solution was further diluted to approximately 1 µg/ml (0446AF14).

Samples 0446AF01-06 were derivatized with heptafluorobutyryl imidazole according to the described procedure (ratio toluene/HFBI = 9/1); derivatization at 60 ºC for 30 min. Subsequently, the samples were washed with water (2 x 0.1 ml), aqueous 0.1 M Na_2CO_3 (0.1 ml), water (0.1 ml) and dried on $MgSO_4$. Sample codes, see Table 3.

Table 3. Sample codes after modified Edman degradation and subsequent HFBI derivatization

Globin sample	Description	Internal standard*	Sample code after modified Edman degradation	Sample code after subsequent derivatization with HFBI
0442AF01	blank	0442AF07; 50 µl	0446AF01	0446AF15
0442AF03	10 nM	0442AF07; 50 µl	0446AF02	0446AF16
0442AF04	100 nM	0442AF07; 50 µl	0446AF03	0446AF17
0442AF05	1 µM	0442AF08; 50 µl	0446AF04	0446AF18
0442AF06	10 µM	0442AF08; 50 µl	0446AF05	0446AF19
0442AF09	d_0/d_8, 5/5 µM	n.a.	0446AF06	0446AF20

* solutions of internal standard globins were used as described in Table 1

(Wednesday, November 10, 2004)

GC-EI-MS analysis

GC-EI-MS analysis on the MAT900 gave too many fragments. Since NICI on the MAT900 is not sensitive enough, it was decided to use a triple quad-GC-MS (TSQ7000), operated by Dr. J. Driskell. First, the 1 µg/ml standard was evaluated; a rather large peak resulted. The fragment with *m/z* 564 was the most abundant fragment; comparable results as obtained by TNO-PML.

Globin sample work-up

Globin samples of accidentally exposed person, 0446DN03 (21.4 mg) and 0446DN04 (20.4 mg), were subjected to modified Edman degradation. Both samples readily dissolved in formamide. Globin 0442AF07 (50 µl of a solution of 25.1 mg in 1.25 ml formamide) was used as an internal standard.

Two globin samples were used for exercising the method: 0442AF01 (blank) and 0442AF05 (1 µM exposed). The procedure as described above was followed. At the end of the day, the samples were concentrated with a Zymark TurboVap Evaporator under heating at 60 ºC by applying a N_2 flow and stored as such at –20 ºC.

Table 4. Globin samples (exercise and "real" samples)

Globin sample	Description	Amount used (mg)	Internal standard*	Sample code after modified Edman degradation and subsequent derivatization with HFBI
0446DN03	"real" sample	21.4	0442AF07; 50 µl	0446AF21
0446DN04	"real" sample	20.4	0442AF07; 50 µl	0446AF22
0442AF01	blank	23.6	0442AF07; 50 µl	0446DA01
0442AF05	1 µM	20.3	0442AF08; 50 µl	0446DA02

*solutions of internal standard globin were used as described in Table 1

LC-tandem MS analyses

The LC tandem MS analyses of samples 0446AF07-12 showed that an exposure level of 1 µM could still be determined; gradient elution of 50/50% CH_3CN/H_2O to 100% CH_3CN was now applied and samples had also been dissolved in CH_3CN/H_2O (50/50, 100µl). The peak of the internal standard was present at a level that could be expected on the basis of concentrations: the internal standard was present at a level equivalent to 5 µM. For a similar transition, the peak for the native adduct in the 10 µM sample was twice as large as for the deuterated compound. Again, the transition of *m/z* 429 (MH^+) → *m/z* 130 was the most intense. The identity of the 130 fragment is yet unknown. Exploratory research using relatively high standards was also performed on the TSQ Quantum LC tandem MS.

(Thursday, November 11, 2004)

GC-NICI-MS analyses

GC-NICI-MS analyses on the TSQ7000 were continued. There were some problems with regard to the peak ratio d0/d8: the d8 level could be observed at the level that could be expected. However, the level of the d0 was much too high, and was also present in the blank. The reason for this was yet unclear. It was decided to use a less steep temperature program for the analyses; also, first the already processed samples 0440AF01-05 will be analyzed. It seemed that by using the temperature program used at TNO-PML the chromatography improved; it seemed as if a 10 nM exposure level could still be detected. Peak ratios d0/d8 were as should be expected.

However, some of the newly prepared samples did not look good, e.g., in 0446AF16 the level of d8-pentafluorophenylthiohydantoin seemed okay, but the level of the native pentafluorophenylthiohydantoin was much too high (see GC-NICI-MS results in annex). Sample 0446AF18 looked okay.

HFB derivatization

Introduction of HFB on exercise samples was performed by CDC scientist according to the described procedure under supervision of TNO scientist.

Introduction of HFB on "real" samples was performed according to the described procedure; resulting sample codes 0446AF21 and 0446AF22 (see Table 4).

Exercising isolation of globin

From blood samples labelled 04-905-0032-SPRB2 (under top label the sample was labelled 04-905-0032-B1; under the latter label was the original label Mould072704) and 04-905-0036-SPRB2 (under top label the sample was labelled 04-905-0036-P1; under the latter label

was the original label Mould082304), was taken 0.5 ml for isolation of globin. Codes of samples to be processed: 0446DN05 (Mould072704) and 0446DN06 (Mould082304); codes of resulting globin samples: 0446DN07 (87 mg; derived from Mould072704) and 0446DN08 (116 mg; derived from Mould082304).

(Friday, November 12, 2004)
GC-NICI-MS analysis of samples 0446AF21 and 0446AF22 did not show the presence of the pentafluorophenylthiohydantoin-HFB derivative. However, no peaks for the internal standard could either be observed.
Analysis of the samples 0446DA01 and 0446DA02 showed only peaks for sample 0446DA02; however the ratio d0/d8 analyte was ½, and should have been 1/5.

3. Demonstration of phosgene method
(Monday, October 17, 2005)
Method will be performed together with the CDC scientists Kerry Preston and Adrian Woolfitt.
Already processed samples 0535AF10 (blank), 0535AF12 (10 microM), 0535AF15 (50 microM) and 0535AF17 (100 microM ^{13}C-phosgene), stored at CDC at –70 °C, were thawed. 0.5 ml of each sample was transferred to 6 ml tubes (without caps; other tubes did not fit in Eppendorf rotor). The samples were lyophilized, after freezing in solid CO_2, using an Eppendorf Vacufuge. Two runs: blank + ^{13}C, then 10 + 50 microM exposed samples. Samples were fully lyophilized after 2 h, giving a white residue. The samples were stored in the freezer (-20 °C) overnight.
In the mean time the LC tandem MS instrument (type: Applied Biosystems MDS Sciex 4000 Q Trap; newly installed instrument) was prepared for analysis of samples, standards etc.
The guanidine buffer was prepared according to the work plan (sent by e-mail to Maria Solano, Tuesday October 11, 2005).

(Tuesday, October 18, 2005)
The lyophilized samples of Monday were treated with DTT and iodoacetic acid (Na+ salt), together with a CDC scientist, according to the work-plan. Deviation from work plan: 50 mg iodoacetic acid, Na+ salt was dissolved in 180 microL guanidine buffer; 40 microL of this solution added to the DTT-reduced sample solutions.
Dialysis started around 13.00 (1 liter 50 mM NH_4HCO_3) per sample. Dialysis buffers refreshed at 15.30 and at 17.00. Continued overnight.
LC tandem MS analysis of already processed sample 0535AF25 (100 microM exposure) was started. No real conclusive results after one injection. Therefore, the reference standard (0511AF01; 10 microgram) was chosen for optimization of the instrument. The sample was dissolved in water containing 0.2% formic acid to an appropriate concentration.

(Wednesday, October 19, 2005)
Samples were removed from dialysis cassettes and digested with trypsin, as described below (*method was not incorporated in work plan*):
Dialyzed (reduced and carboxymethylated) albumin was transferred from the slide-a-lyzer cassettes into vials (4 mL). Next, trypsin was added (2 % w/w). For this purpose, trypsin (6 mg) was dissolved in buffer (5 mL, 50 mM NH_4HCO_3). Part (50 microliter) was added to the dialyzed solution of reduced and carboxymethylated albumin in buffer. Incubation was performed at 37°C for 4 h. Finally, the sample was filtrated through a pre-rinsed (50 mM

NH_4HCO_3, 3x 1 mL) filter with a 10 kDa cutoff with centrifugation at 3200g, to remove the enzyme.
The resulting trypsin digests were coded as follows:

Blank:	0542DN01
10 microM exposure:	0542DN02
50 microM exposure:	0542DN03
^{13}C-labelled exposure:	0542DN04

Two samples (0535AF10 (blank), and 0535AF15 (50 microM exposure)) were selected for practicing the method. The procedure as described above was performed, starting with lyophilization.

(Thursday, October 20, 2005)

Samples 0542DN01-04 had been analyzed overnight. The 10 microM (0542DN02) level could not be distinguished from the blank; in the 50 microM (0542DN03) level the adduct could be detected. The sensitivity of instrument was further improved.
The CDC scientist trypsinized the two dialyzed samples, according to the work plan. Changes injection volume to 50 microL. The results looked much better then.
Codes of trypsin digests:

Blank:	0542KP01
50 microM:	0542KP02

These samples will be analyzed by means of LC tandem MS.

(Friday, October 21, 2005)
Analyses of the phosgene bis-peptide adduct looked nice. The recorded spectra were compiled and the project closed.

4. Demonstration of lewisite method

(Monday, October 17, 2005)
Method will be performed together with CDC scientists Maria Solano and Joe Wooten.
Instrument to be used: MAT 900 XL. The GC-column was installed (type: Varian CP-Sil 5CB, 50 m length, 0.25 mm i.d., 0.25 mm film thickness)
Start with already processed samples 0536AF13-17. These samples had already been analyzed at TNO; it seemed as if the vial-inserts had been leaking their constituents into the vial. For this reason, 20 microliter of toluene were added to each sample. Although the absolute intensity of the signals will now definitely decrease, the ratios between analyte and internal standard should not be different. The first sample that was analyzed was 0536AF17 (10 microM exposure). Satisfactory result: see file C:\Xcalibur\..\Oct,05\mo529001. The other samples were run overnight.
Since not all reagents had arrived at CDC, the work up of samples 0536AF08-0536AF12 was to performed at Tuesday (deviation from work plan).

(Tuesday, October 18, 2005)
Analytical results obtained from overnight GC-MS runs: looked okay at first sight; chromatograms need still to be integrated. After a second look, the peak ratio of the analysis of 0536AF16 gave the same result as 0536AF17 (both ca 7). Probable course might have been a switch of samples. All samples will be reanalyzed (see Thursday).

Stock solutions were prepared for BAL (100 mM and PAB (100 microM), according to work plan.
From the exposed, washed and lysed erythrocyte samples 0536AF08-12, 1 ml was taken for processing. The rest was transferred into new vials and stored at –70 °C.
New codes for samples to be processed:
0542AF01 (1 ml from 0536AF08)
0542AF02 (1 ml from 0536AF09)
0542AF03 (1 ml from 0536AF10)
0542AF04 (1 ml from 0536AF11)
0542AF05 (1 ml from 0536AF12)

To these samples (0542AF01-05), 10 microL of BAL solution and 10 microL of PAB solution were added and the samples were incubated for 4 h (deviation from work plan; not relevant). The samples were diluted with water (9 mL). Sep-Pak C18 cartridges were conditioned according to the work plan. The samples were applied and the cartridges were eluted according to the work plan. For each sample, the appropriate fraction was isolated and concentrated; due to the unavailability of an appropriate device for concentrating samples in the way it is performed at TNO, the samples were concentrated under nitrogen in a special device (TurboVap evaporator). The remaining water was removed by concentration in the Vacufuge. The blank was concentrated in two steps; step 1: volume reduction using a stream of air; step 2: concentration in Vacufuge.
For future work this week, it was decided to split the samples (organic layer from aqueous layer) and to concentrate them separately.

(Wednesday , October 19, 2005)
Samples 0542AF01-05 were concentrated to dryness and coevaporated twice with toluene (0.5 mL). Next, the samples were dissolved in toluene (100 microliter) and transferred to a 0.5 mL GC vial with a conically shaped bottom. Heptafluorobutyryl imidazole (10 microlitres) were added and the reaction mixtures were incubated for 1 h at 50° C. It should be mentioned that the HFBI used was a red troubled liquid (had been stored in refrigerator). Next, the reaction mixtures were washed with water (100 microliter), dried over $MgSO_4$ in a pasteur pipette, and analyzed with GC/MS.
The remaining, washed, exposed erythrocytes (samples 0536AF08-12) were divided in two portions of 1 mL. To one set of these samples (coded as 0542JW01-05), 10 microL of BAL solution and 10 microL of PAB solution were added and the samples were incubated for 1h according to the work plan. Next, work-up was performed according to the work plan. The eluate, consisting of two layers, was now divided into two separate fractions in order to speed up the concentration step. The upper (aqueous) layer was concentrated in the Vacufuge, while the lower (organic) layer was concentrated to a small volume using a stream of nitrogen. Finally both fractions were combined again, evaporated to dryness, and coevaporated twice with toluene (0.5 mL). It was decided to postpone the derivatization with HFBI to Thursday, using newly ordered HFBI.
The set of samples (0536AF13-17) prepared in The Netherlands was reanalyzed; unfortunately, sample 0536AF15 did not contain enough material to be analyzed again. The set of samples (0542AF01-05), prepared at CDC, was also analyzed.
In case of 0536AF13-17, the peak ratios now corresponded with the peak ratios found at TNO. Since the samples had been diluted with toluene on Monday (there was not enough sample anymore, probably due to leakage of the insert in the vial), the lowest exposure level

that could be analyzed was approximately 20 nM. The sample (0536AF16) that showed a deviating peak ratio on Tuesday, was now okay.
The other set (0542AF01-05) gave low responses, probably due to the derivatization that did not proceed satisfactory (bad quality of HFBI; see Wednesday). Even the 100 nM exposure level (0542AF03) could not be detected anymore. The 1 microM exposure (0542AF04) gave a peak ratio analyte/internal standard that corresponded with earlier analyses. The 10 microM exposure level, however, showed a deviating peak ratio (19 instead of 6 – 7).

(Thursday, October 20, 2005)
A CP-Sil 5b column was installed in GC/MS (exactly the same type as was used for the analysis at TNO) of J. Wooten in order to get a mirror view of the results we obtained at TNO.
The MAT 900 XL was further optimized in order to improve the sensitivity.
Fresh HFBI (Pierce) had arrived; colourless and clear liquid (at room temp). Processed samples 0542JW01-05 were derivatized by CDC scientist with fresh HFBI, according to the work-plan, and were worked up further.
Codes after derivatization:
0542JW01 → 0542JW06 (blank)
0542JW02 → 0542JW07 (10 nM)
0542JW03 → 0542JW08 (100 nM)
0542JW04 → 0542JW09 (1 microM)
0542JW05 → 0542JW10 (10 microM)

Unfortunately, layer separation appeared difficult this time, using the conically shaped GC vials. When the toluene layer was extracted with water (4 x 0.1 mL) each time the water layer was collected in a tube. When performing the drying step, it appeared that most of the liquid consisted of water. So it was decided to add 100 microL of toluene to the collected water extracts, and dry that again over $MgSO_4$.
Analysis showed the expected peaks only for sample 0542JW10; the intensity of the peaks was, however, much lower than for the corresponding sample 0542AF05. The reason for this is yet unknown.

IV RESULTS

IV.1 Demonstration of the SOP for the albumin-tripeptide assay for detection of sulfur mustard exposure

Background

In 1999, Noort et al published a sensitive method for detecting exposure to sulfur mustard based on pronase digestion of albumin, and subsequent mass spectrometric analysis of the resulting tripeptide Cys*-Pro-Phe, with Cys* the sulfur mustard-modified Cys-34 residue of albumin (Noort et al., 1999).

This assay was demonstrated to a scientist of USAMRICD in 2004, as was described in the previous annual report. The actual demonstration was performed at CDC; consequently, a CDC scientist took also part in the demonstration.

Method demonstration

Especially the use of affinity chromatography for isolation of albumin was further explored. Also, the automation of the microliquid chromatography – electrospray – tandem mass spectrometric analysis was initiated. The demonstration has been described in detail in the previous annual report and has also been published as a joint publication (Noort et al., 2004b).

In the next grant period, attention will be paid to further automation of this methodology, i.e. by performing the enzymatic digestion on-line by means of immobilized pronase. For this purpose, we will collaborate with the group of Prof. Dr. Irth (Free University, Amsterdam, The Netherlands), as is already the case for the phosgene method (see below).

IV.2 Demonstration of the modified Edman degradation of sulfur mustard modified hemoglobin

Background

Sensitive methods have been reported for sulfur mustard adducts with proteins. In case of the N-terminal valine globine sulfur mustard adduct, the alkylated amino acid can be selectively cleaved using a method developed for other alkylating agents (Törnqvist et al., 1996). Reaction of globin with pentafluorophenyl isothiocyanate releases the alkylated amino acid as the hydantoin, which is further derivatized to its heptafluorobutyryl derivative and analyzed by GC-MS or GC-MS/MS (Fidder et al, 1996; Noort et al., 2004a). The actual chemistry on which the modified Edman degradation is depicted in Figure 1.

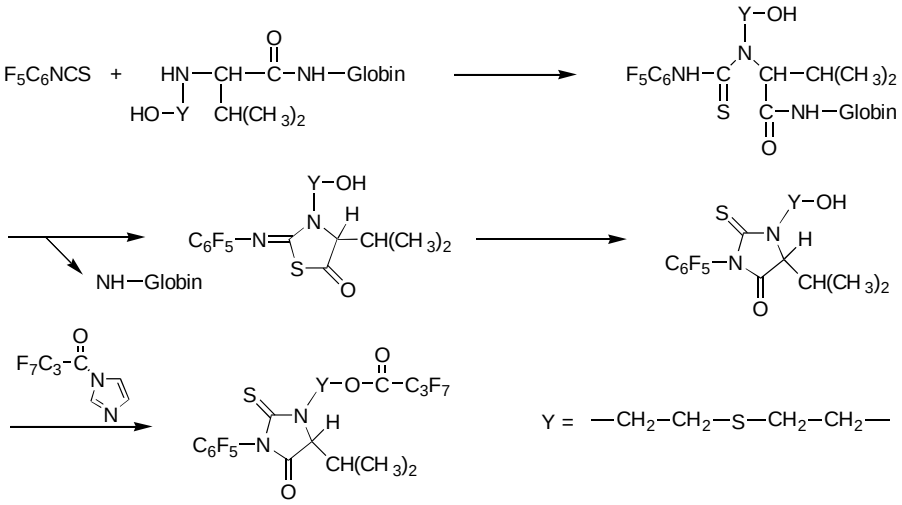


Figure 1. Modified Edman degradation of sulfur mustard-modified globin

Method demonstration

The method demonstration was focused on two objectives. The first objective was to see whether the method for modified Edman degradation of sulfur mustard-modified globin and subsequent GC-MS analysis could be installed within a relatively short period (4 days) of time. The second objective was to evaluate the use of LC tandem MS analysis for analysis of the underivatized pentafluorophenylthiohydantoin derivative obtained after modified Edman degradation.

A batch of underivatized pentafluorophenylthiohydantoin was synthesized in our laboratory, starting from synthetic N-terminal valine - sulfur mustard adduct (see Figure 2 for GC-MS analysis).

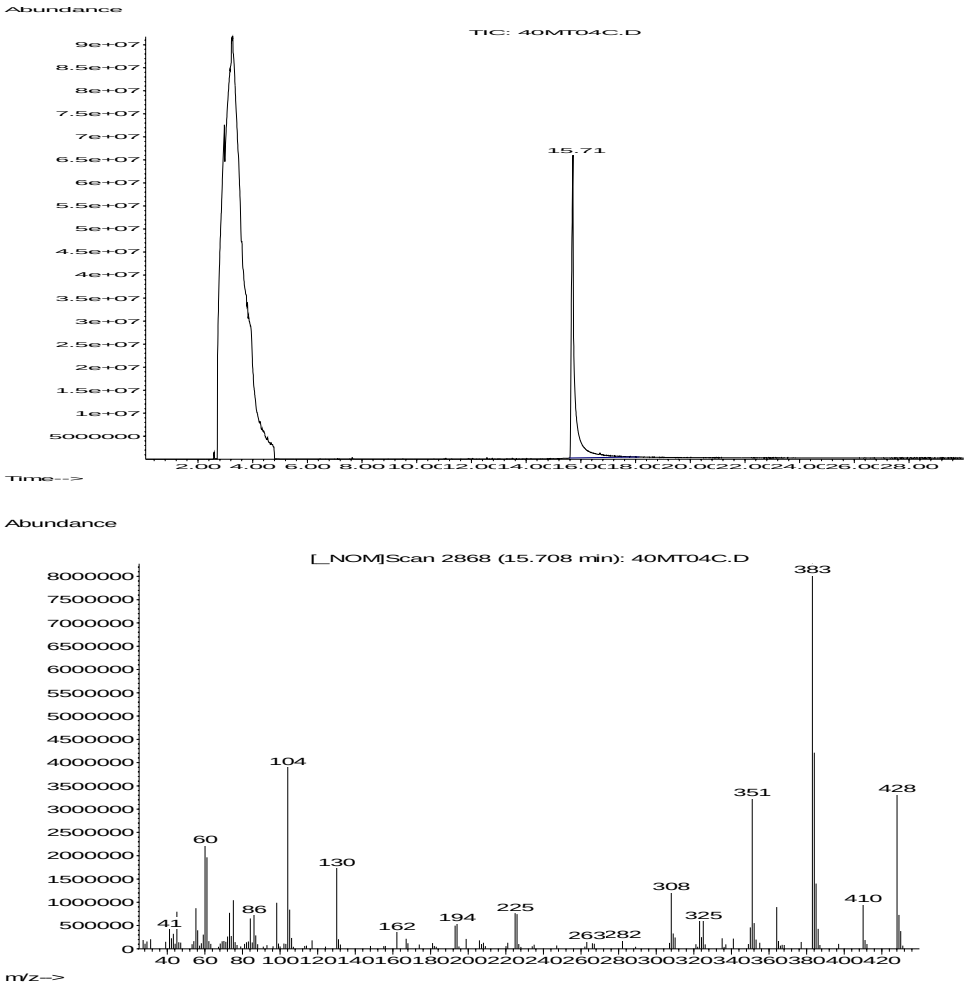


Figure 2. GC-MS analysis of pentafluorophenylthiohydantoin derivative of sulfur mustard adduct of N-terminal valine; upper trace GC-MS chromatogram; lower trace, EI-MS spectrum.

With regard to the first objective, it turned out that GC-EI-MS analysis of the pentafluorophenylthiohydantoin gave too many fragments. Since NICI analysis on the MAT900 is not sensitive enough, it was decided to use a triple quad-GC-MS (TSQ7000). The synthetic reference standard, after derivatization with HFBI, gave the expected mass spectrum after GC-NICI-MS; comparable spectra were obtained. For the already processed samples, a 10 nM level could still be detected. This is an improvement of the analyses performed at TNO, since in the latter case only a 100 nM exposure level could be detected. With regard to newly prepared samples, i.e. samples processed at CDC by a CDC-worker and a TNO-worker, rather variable results were obtained which might lie in the fact that concentration of processed samples was performed by heating under a stream of dry nitrogen and that relatively high amounts of reference materials were used, during work-up of relatively low-level exposed globin samples.

With regard to the second objective, it turned out that LC tandem MS analysis can be applied for analysis of the specific pentafluorophenylthiohydantoin derivative to globin samples, processed according to the modified Edman degradation. The transition m/z 429 → 130 was selected for MRM (see Figures 3 and 4).

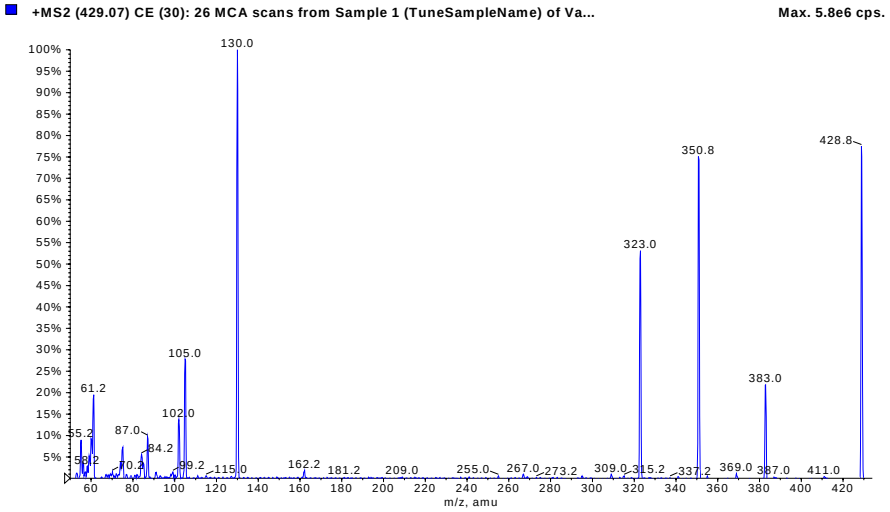


Figure 3. ESI/MS/MS product ion spectrum of pentafluorophenylthiohydantoin derivative of N-terminal valine adduct of sulfur mustard. This is a composite spectrum, created during automatic optimization, using a range of collision energies.

It turned out that the native standard had a rather long retention time (30 min) when a gradient of 99% water/1 % formic acid to 80%/20% CH_3CN, 1% formic acid was applied. Also, we suspected that the solubility of the pentafluorophenylthiohydantoin in aqueous solutions would be a problem. Best results were obtained with a gradient from CH_3CN/water, 1/1, 1% formic acid to 98% CH_3CN, 1% water, 1% formic acid, when the compound was dissolved in CH_3CN/water, 1/1, 1% formic acid.

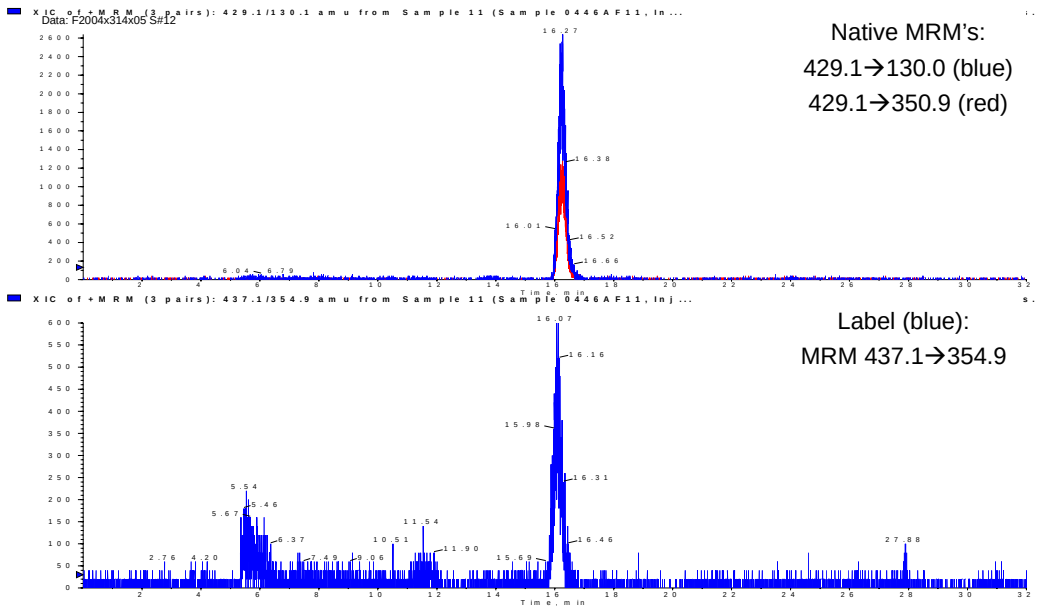


Figure 4. LC tandem MS analysis of a processed globin sample from blood that had been exposed to sulfur mustard. The upper trace represents the analysis of the native adduct (1 µM exposure level), and the lower trace represents the analysis of the d_8-deuterated adduct (5 µM exposure level).

The compounds appear to give relatively poor ionization by positive ion electrospray. The signal strength is much weaker than we routinely observe with albumin sulfur mustard tripeptide adducts. MS/MS fragmentation is good, with 3 or 4 strong ions to choose from. The estimated detection limit for the valine adducts is > 0.1 µM exposure level.

IV.3 Demonstration of the method for diagnosis of exposure to phosgene

Background

Phosgene is a highly reactive nucleophile with a half-life < 1 s in water. With regard to biological fate, it has been reported that phosgene reacts with glutathione to form a bis-conjugate (Fabrizi et al., 2000), and with cysteine to form 2-oxothiazolidine-4-carboxylic acid (Kubic and Anders, 1980). Also, it can react with the polar heads of phospholipids (Di Consiglio et al., 2001). Phosgene has also been reported to bind to haemoglobin and albumin (Noort et al., 2000). During exposure of human serum albumin to phosgene the lysine 195 and 199 residues are bridged, under formation of a urea-type bridge. Trypsin digestion of the phosgene-modified albumin results in the formation of a bis-peptide containing the normal tryptic fragments T25-T26 and T27-T28, with T25 and T27 linked by a carbonyl group (see Figure 5; Noort et al., 2000).

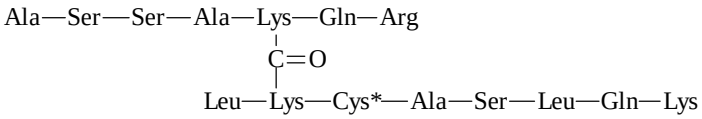


Figure 5. Structure of the phosgene adduct (ASSAK*QR)(LK*ZASLQK), with a C=O bridge between K* residues and with Z as carboxymethylcysteine [O=C-(T25)(T28)]

Method demonstration

The method was demonstrated to CDC workers as described in the annex of the experimental part. In advance of the actual demonstration, a number of reference compounds, already processed samples, and (exposed) samples to be processed at CDC had been sent to CDC. It appeared that the method could be relatively easy be transferred, having in mind that the instrument (API 4000 Qtrap) had been installed only one week prior to the demonstration (see Figure 6 for a representative run).

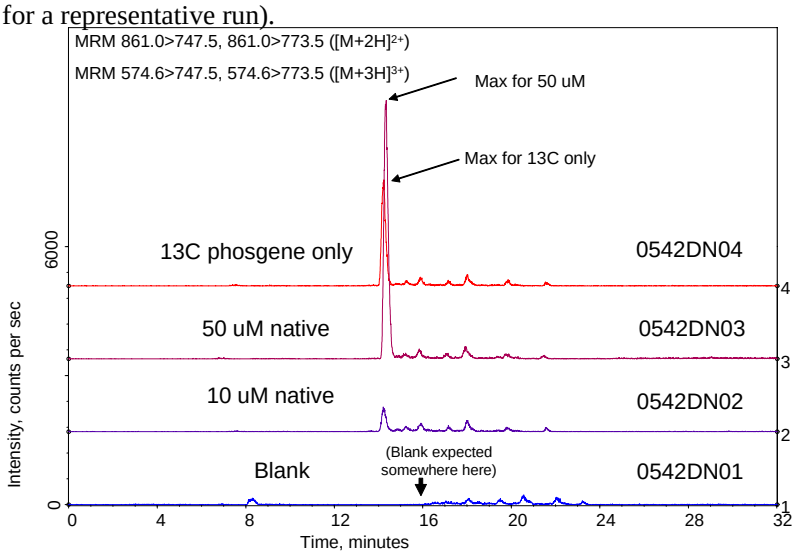


Figure 6. Tandem MS analysis of tryptic digests of albumin from ^{13}C-phosgene and normal phosgene-exposed plasma samples; MRM traces were summed.

From Figure 6 it became obvious that the ^{13}C-labelled internal standard cannot be used due to crossover of signals. Analogously, when the transition MRM 861.7 → 774.5 (i.e. the preferred MRM for the internal standard derived peptide adduct) was monitored, crossover was also observed for the native peptide adduct. As was also the case during earlier analyses (Noort et al., 2000), no real clean blank could be observed (see Figure 7). The reason for this is still unknown, but probably the presence of large amounts of carbon dioxide (i.e.,"inactivated phosgene") may play a role in it.

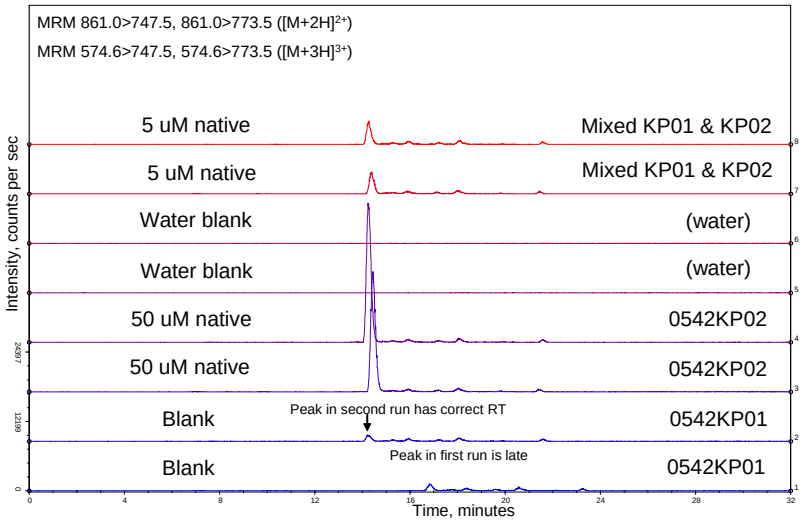


Figure 7. Tandem MS analysis of tryptic digests of albumin from phosgene-exposed plasma samples; MRM traces were summed. These particular samples were processed independently by a CDC worker.

Synthesis of reference peptide O=C-(T25-T28)

The most pragmatic way for the synthesis of the reference peptide is by reaction of phosgene with the two individual peptides Ala-Ser-Ser-Ala-Lys-Gln-Arg and Leu-Lys-Cys(S-carboxymethyl)-Ala-Ser-Leu-Gln-Lys. In that case, we should deal with the following. First, certain functional groups (N-terminal amino-groups, the NH_2 of the C-terminal lysine residue and the arginine group) have to be protected, since otherwise too many side-products will be formed, giving a complex, probably inseparable mixture of products. Second, in any case, symmetrical products will be formed, such as (Ala-Ser-Ser-Ala-Lys*-Gln-Arg)$_2$-C=O and (Leu-Lys*-Cys(S-carboxymethyl)-Ala-Ser-Leu-Gln-Lys)$_2$=O, with Lys* the site of carbonylation.

Having this in mind, we envisaged that the following peptide building blocks had to be synthesized:

1. (Z-Ala)-Ser-Ser-Ala-Lys-Gln-Arg(NO_2)
2. (Z-Leu)-Lys-Cys(S-carboxymethyl)-Ala-Ser-Leu-Gln-(Lys-Z)

Schematically, the synthesis proceeds as depicted in Figure 8.

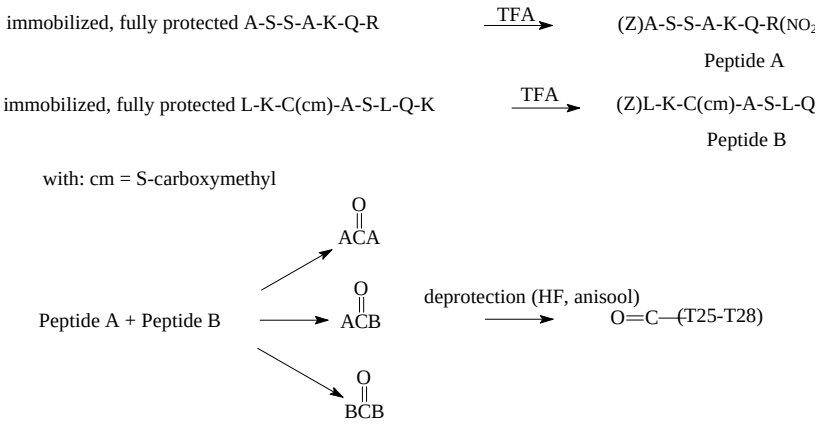


Figure 8. Synthetic methodology towards phosgene – albumin biomarker peptide.

The benzyloxycarbonyl (Z)-group was selected as protective group for the N-terminal amino functions, for the ε-NH_2 group of lysine and for the nitro group of the guanidine moiety in arginine. These protective groups are resistent against trifluoroacetic acid required for splitting the peptide from the resin. The peptides were further synthesized by means of standard Fmoc solid phase peptide chemistry, i.e., with acid-labile protective groups for the other side chains.

The particular cysteine building block, which was not commercially available at that time, was prepared by reaction of cysteine with tert-butyl bromoacetate, followed by reaction with Fmoc-Cl. Fmoc-Lys(Z) and Fmoc-Arg(NO_2) were coupled to SAC-Tentagel gekoppeld. The desired resins were obtained in good yield. The capacity of the resins was 18 µmol/100 mg for NH_2-Arg(NO_2) and 24 µmol/100 mg for NH_2-Lys(Z)-resin. The peptides were synthesized according to standard protocols on a solid-phase peptide synthesizer. Cleavage from the resin was effected by treatment with trifluoroacetic acid/water, 95/5, v/v.

The partially protected peptides were purified by means of semi-preparative reversed phase HPLC. Subsequently, they were linked with a carbonyl bridge through reaction with phosgene at pH 9. Except for the desired compound, also two symmetrical products were formed, according to LC tandem MS. The desired, partially protected bis-peptide was isolated and purified by means of semi-preparative reversed phase HPLC; see Figure 9 for MS spectrum.

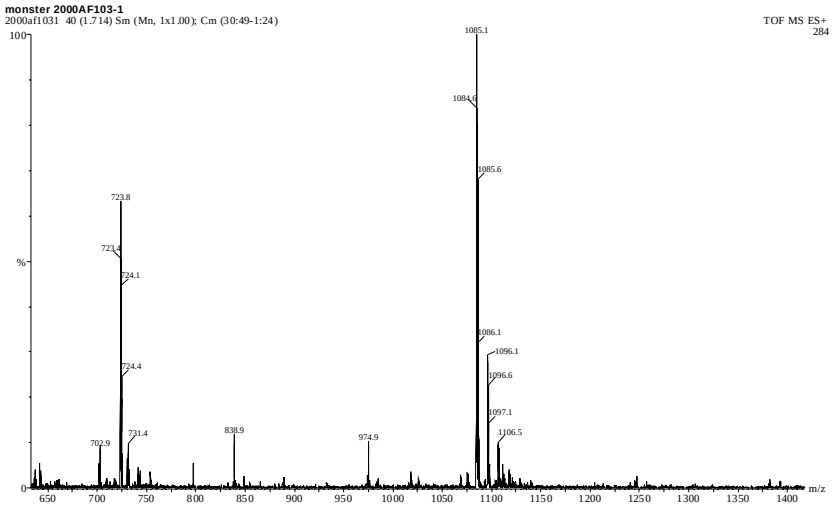


Figure 9. Electrospray MS spectrum of partially protected, synthetic O=C-(T25-T28): ((Z-Ala)-Ser-Ser-Ala-Lys*-Gln-Arg(NO_2))---(C=O)---((Z-Leu)-Lys*-Cys(S-carboxymethyl)-Ala-Ser-Leu-Gln-(Lys-Z), with Lys* as Lys-195 and Lys-199 of human serum albumin.

Subsequently, the product was deprotected. Deprotection with BBr_3/TFA resulted in a non-defined product. Treatment with HF.pyridine resulted only in removal of the Z-groups; the NO_2 group remained on the Arg residue. Deprotection with HF/anisole afforded the desired deprotected product. The product was analyzed by means of LC tandem MS and had identical retention time and MS spectrum as the bis-peptide adduct obtained after trypsin digestion of phosgene-modified albumin (see Figure 10).

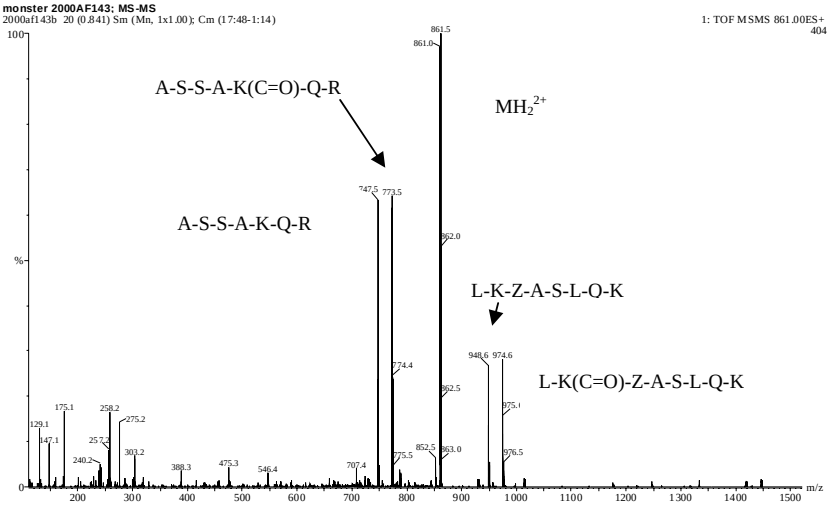


Figure 10. Electrospray tandem MS spectrum of synthetic, fully deprotected O=C-(T25-T28).

IV.4 Approach for automation of the assay for diagnosis of phosgene exposure

In collaboration with the group of Prof. Dr. Irth of the Free University of Amsterdam (The Netherlands), we have explored the possibilities to automate certain steps in the assay for

diagnosis of phosgene exposure. To this end, the approach depicted in Figure 11 will be followed:

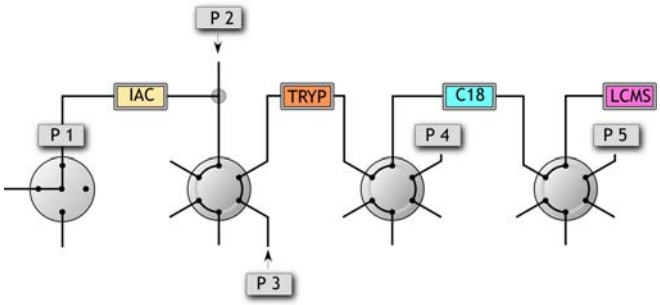


Figure 11. Analytical approach towards automation of phosgene assay; IAC, immunoaffinity chromatography for albumin; TRYP, immobilized trypsin; C18, C18 SPE cleanup.

In the first step, albumin will be isolated from plasma by immunoaffinity chromatography. It is believed that this will give a more pure sample than isolation by normal affinity chromatography. In the second step, the albumin will be enzymatically cleaved on a column containing immobilized trypsin. Subsequently, the digest will be reconcentrated on a short C18 SPE column, after which the sample will be analyzed by means of LC-tandem MS. From the above scheme it is obvious that the carboxymethylation step has been omitted. Until now, it is not yet possible to perform a carboxymethylation step on-line. So, we should either focus on a slightly different biomarker that does not contain the carboxymethylated cysteine residue, or the carboxymethylation should be performed off-line.
Until now, attention has been focused on automation of the C18 SPE desalination of the synthetic adduct. The peptide could readily be analyzed, giving identical mass spectral data.
We continued with the immunoaffinity isolation of modified HSA from plasma, followed by the off-line sample handling, i.e., sample lyophilization, carboxymethylation, and trypsin digestion. Finally, the sample was analyzed with SPE-LC-MS analysis, as described above. The following results were obtained (see Figure 12); in this case, 30 µl of the plasma sample was processed.

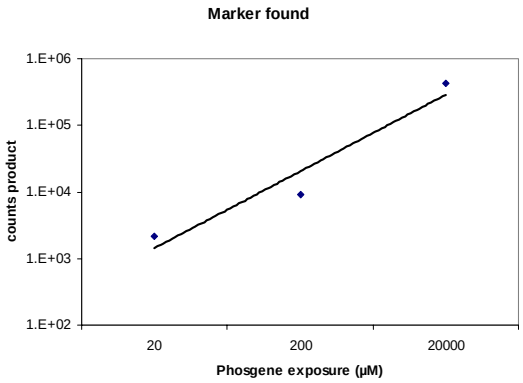


Figure 12. Semi-quantitative dose-effect relationhip of formed O=C-(T25-T28) in processed albumin, isolated from plasma that had been exposed to various concentrations of phosgene. Sample work-up consisted of albumin isolation by immunoaffinity chromatography, and off-line sample handling, i.e., sample lyophilization, carboxymethylation and trypsin digestion.

IV.5 Demonstration of the method for diagnosis of exposure to Lewisite

Background

Trivalent arsenic has a high affinity for thiol groups. Upon incubation of human blood with ^{14}C-lewisite, 93% of the total radioactivity was found in the erythrocytes, with 25-50% associated with globin (Fidder et al., 2000). LC tandem MS of tryptic digests indicated the presence of several binding sites, and specifically identified a crosslink between the cysteine-93 and cysteine-112 residues of β-globin. The affinity for thiols has also been exploited in analytical methods. We use an analytical method that employs 2,3-dimercapto-1-propanol (BAL) for displacement of bound lewisite residues or hydrolyzed lewisite (CVAA) residues (see Figure 13). The resulting derivative is further derivatized with a heptafluorobutyryl group, which was initially meant to enable GC-NICI analysis. Unfortunately, the HFB group was easily split off under NICI conditions. However, the HFB derivative enabled sensitive analysis under EI conditions, allowing the detection of exposure levels of > 1 nM (see Fidder et al, 2000).

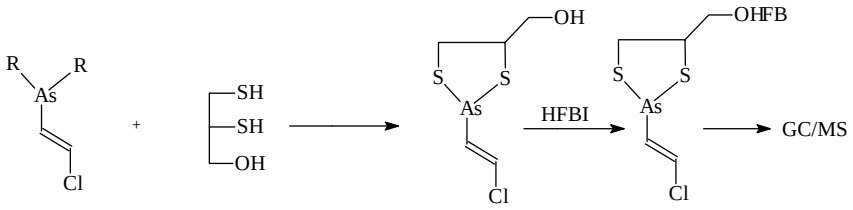


Figure 13. Chemical structures of dichloro(2-chloorvinyl)arsine (Lewisite, L1), 2-chlorovinylarsonzuur (CVAA), British Anti-Lewisite (BAL) and conversion into CVAA-BAL-HFB derivative.

Demonstration

The already processed samples that were sent to CDC gave almost identical analytical results, as obtained at TNO. Also, the reference standards that had been prepared could be analyzed satisfactorily. However, work-up of samples at CDC gave rather variable results, which could mainly be attributed to slightly different conditions, materials and chemicals that were used. Also, the instrument we used had not been calibrated with the synthetic standard in advance of the actual method demonstration. It is recommended for future work to reserve a longer time period than one week for the demonstration.

IV.6 Development of a rapid assay for diagnosis of exposure to cholinesterase inhibitors

Background

The biochemical target for nerve agents is the enzyme acetylcholinesterase (AChE). Nerve agents inhibit this enzyme by phosphylation of a serine hydroxyl group within the active site. A similar reaction will occur with the related enzyme human butyrylcholinesterase (HuBuChE). AChE and HuBuChE, which have half-lives of 5-16 days, provide excellent biomarkers. HuBuChE is usually preferred to AChE because it is much more abundant in blood plasma than AChE in erythrocytes and more easy to isolate.

A rather straightforward method for diagnosis of exposure to nerve agents comprises the displacement of the bound organophosphorus moiety by incubation with a large excess of fluoride ion, under formation of an alkyl methylphosphonyl fluoridate (e.g. Degenhardt et al., 2004). However, this method is not suitable for soman and for poorly reactivatable compounds.

A more versatile, but also more laborious method of detection involves isolation of HuBuChE from plasma using affinity SPE, digestion with pepsin, and LC-tandem MS detection of a phosphylated nonapeptide (Fidder et al., 2002). This method surpasses the limitations of the fluoride reactivation assay.

In the current grant period, some experiments have been performed in order to study the recovery of HuBuChE during procainamide affinity extraction. Human plasma (1 mL) was loaded on a cartridge that was filled with 2 mL procainamide gel. After equilibration for 30 min, the gel was washed with 5 or 10 ml 20 mM phosphate buffer and 5 or 10 ml 150 mM NaCl in phosphate buffer. Finally, HuBuChE was eluted with 7 ml 600mM NaCl in phosphate buffer. The HuBuChE activity in each fraction was measured with the Ellman assay. The HuBuChE activity in the plasma sample was also measured using the Ellman assay and the amount of HuBuChE (activity x volume) was set at 100%.

Table 5. Recovery of HuBuChE isolation from human plasma using procainamide affinity extraction (n=2)

Fraction	Volume (mL)	Recovery	Volume (mL)	Recovery
1	5	0 %	10	0 %
2	10	7.8 % ± 0.6	5	1.8 % ± 1.3
3	7	76 % ± 4.2	7	81.0 % ± 4.2
4	5	3 % (n=1)	5	1.4 % (n=1)
Total		87 % ± 4.8		84.2 % ±5.5

Fraction 1: 20 mM phosphate buffer
Fraction 2: 20 mM phosphate buffer + 150 mM NaCl
Fraction 3: 20 mM phosphate buffer + 600 mM NaCl
Fraction 4: 20 mM phosphate buffer + 600 mM NaCl

The data in Table 5 show that the recovery of HuBuChE in fraction 3 ranges between 76-81%. The amount of HuBuChE in fraction 2 is significantly higher when the amount of 150 mM NaCl wash solution was doubled. The higher elution strength of this buffer will probably ensure a better sample cleanup but the recovery of HuBuChE will be lower as well. In order to maximize the recovery of HuBuChE in fraction 3 it is advised to use only 5 ml of 150 mM NaCl-phosphate buffer. Fraction 4 contained only negligible amounts of HuBuChE which means that it is not useful to increase the volume of elution buffer (600mM NaCl in phosphate buffer) in order to improve the recovery of HuBuChE.

Table 6. Recovery of HuBuChE isolation from human plasma using procainamide affinity extraction (n=4)

# ml gel	1	2
Fraction		
1	0.6 % ± 0.3	0.8 % ± 0.2
2	6.3 % ± 1.8	3.0 % ± 0.5
3	68.5 % ± 3.5	83.2 % ± 3.5
Total	75.4% ± 5.6	87 % ± 4.2

Fraction 1: 5 mL, 20 mM phosphate buffer
Fraction 2: 5 mL, 20 mM phosphate buffer + 150 mM NaCl
Fraction 3: 7 mL, 20 mM phosphate buffer + 600 mM NaCl

Table 6 shows the effect of the amount of procainamide gel in the cartridge. The recovery of HuBuChE in fraction 3 is higher using the 2-mL procainamide gel cartridge than using the 1-mL gel filled cartridge. Table 6 shows also that the amount of HuBuChE in fraction 2 is higher in the cartridge that was filled with 1 mL procainamide gel, which means that 1 ml of procainamide gel is the minimum amount to ensure sufficient retention of HuBuChE.

It is remarkable that the total recovery of all fractions is lower than 90%. It must be emphasized that the recovered HuBuChE was present in a high salt buffer. The high salt concentration might interfere with the reaction of the Ellman assay. Preliminary experiments revealed that the measured HuBuChE activity was 10% lower when HuBuChE was dissolved in high salt buffer, i.e. 600 mM NaCl. The recoveries of HuBuChE in fraction 3 shown in the tables are therefore underestimated.

All experiments were performed using the same batch of procainamide gel. It is advised to perform these recovery studies every three months and also once a fresh prepared gel is going to be used, because various or aged batches may show different retention characteristics.

Although the recovery of HuBuChE is rather optimized, the other purpose of this sample preparation step, i.e., sample cleanup, remains underexposed. The extent of sample cleanup can be evaluated by the study of the LC-MS/MS chromatograms, in which the signal to noise ratio needs to be optimized and the chromatograms must be true negative for non-exposed HuBuChE.

It is still under investigation whether the extent of sample cleanup can be further improved by additional sample preparation steps such as ethanol precipitation and or albumin removal. Preliminary results showed that an ethanol precipitation step did not have a significant effect on the analytical results. In this respect, we will also evaluate affinity SPE cartridges that are used for clean-up of plasma samples for proteomics studies.

Figure 14 shows the LC-MS/MS ion chromatogram of a pepsin digest of HuBuChE that was isolated from 100% sarin-inhibited human plasma, following the fast method of HuBuChE isolation, using only 2 ml of procainamide gel. Figure 15 shows the corresponding MS/MS spectrum. It is estimated that approximately 5% inhibition can still be detected according to this methodology.

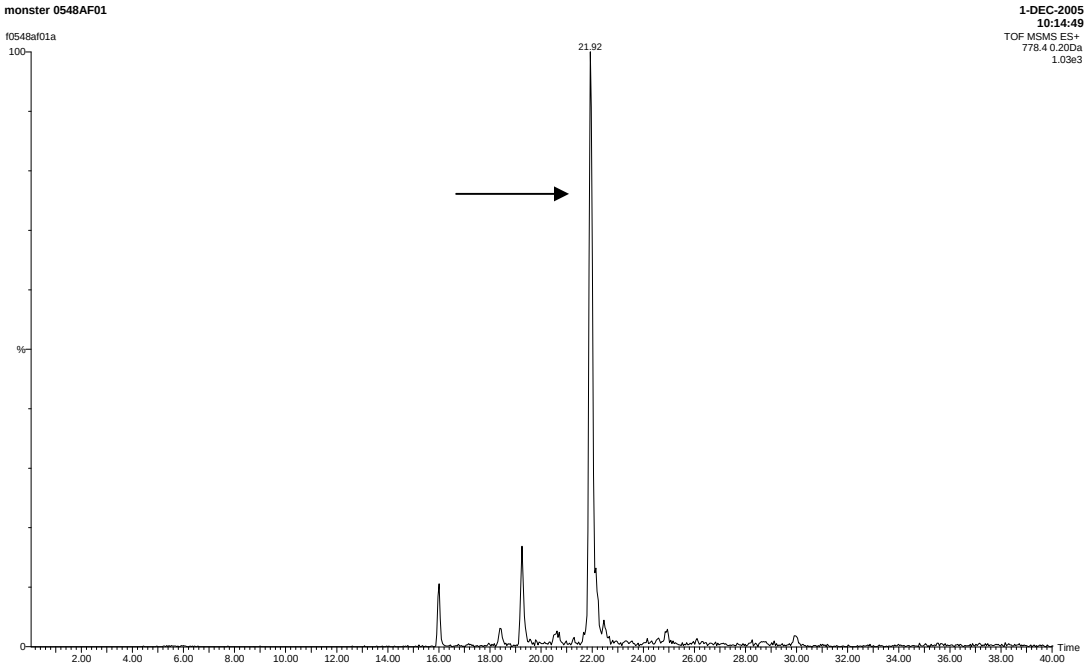


Figure 14. Ion chromatogram of pepsin digest of 100% sarin-inhibited HuBuChE isolated from human plasma. MS/MS conditions: m/z 916 → 778.4. The arrow indicates the peak of the phosphylated nonapeptide.

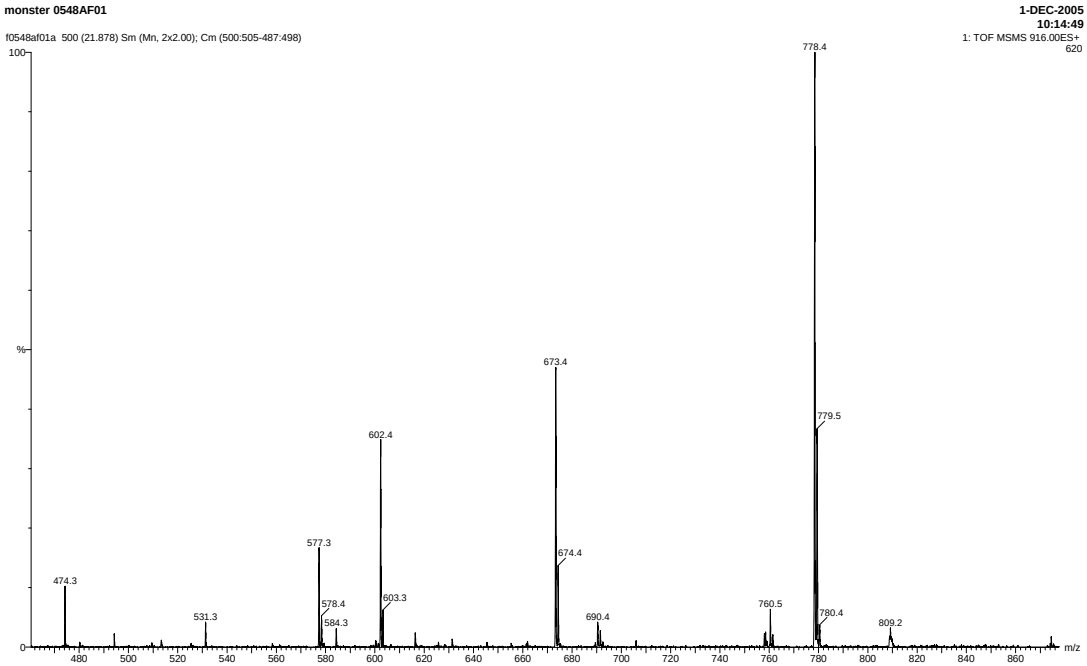


Figure 15. Product ion spectrum of molecular ion MH^+ (*m/z* 916) of nonapeptide FGES*AGAAS derived after pepsin digestion of sarin inhibited HuBuChE isolated from human plasma.

For internal standard purposes, it is proposed to use d_7-sarin, i.e. d_7-isopropyl methylphosphonofluoridate. The advantage of using sarin is that it does not age very rapidly

after inhibition of HuBuChE. Also, the mass difference is large enough to circumvent cross-over with non-labelled sarin. We prefer to use plasma fully inhibited with d_7-sarin as the actual internal standard to be added to the "unknown" sample; a similar strategy was followed in the albumin tripeptide assay for diagnosis of exposure to sulfur mustard. In Figures 16 - 18 the use of d_7-sarin inhibited plasma is exemplified.

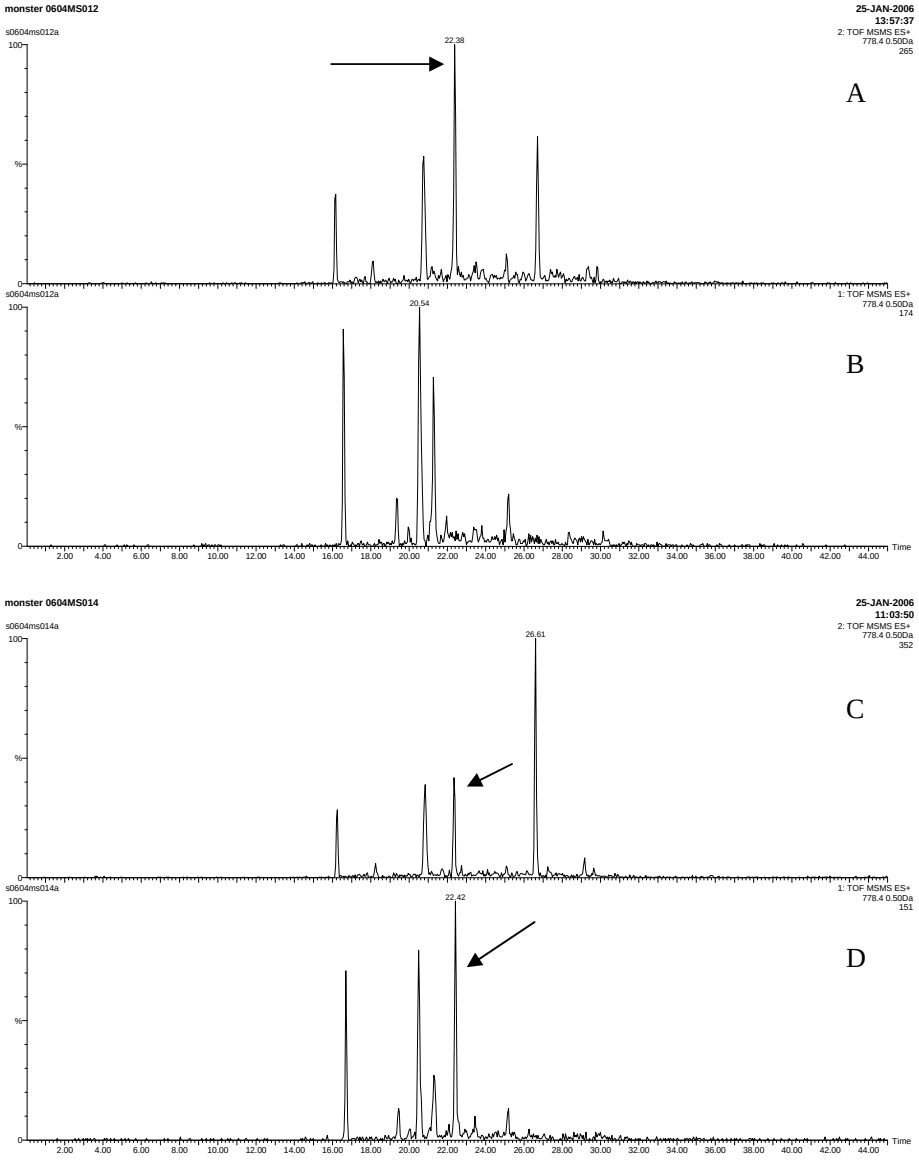


Figure 16. LC tandem MS analysis of FGES(IMPA)AGAAS in pepsin digest of HuBuChE isolated from human plasma; comparison of blank sample (trace A + B) and sarin-exposed sample (trace C and D), in presence of internal standard (plasma exposed to d7-sarin).
The arrows indicate the peaks of interest.

Trace A: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 923.4 (MH^+); d_7-sarin (blank)

Trace B: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 916.4 (MH^+); d_0-sarin (blank)

Trace C: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 923.4 (MH^+); d_7-sarin (exposed)

Trace D: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 916.4 (MH^+); d_0-sarin (exposed)

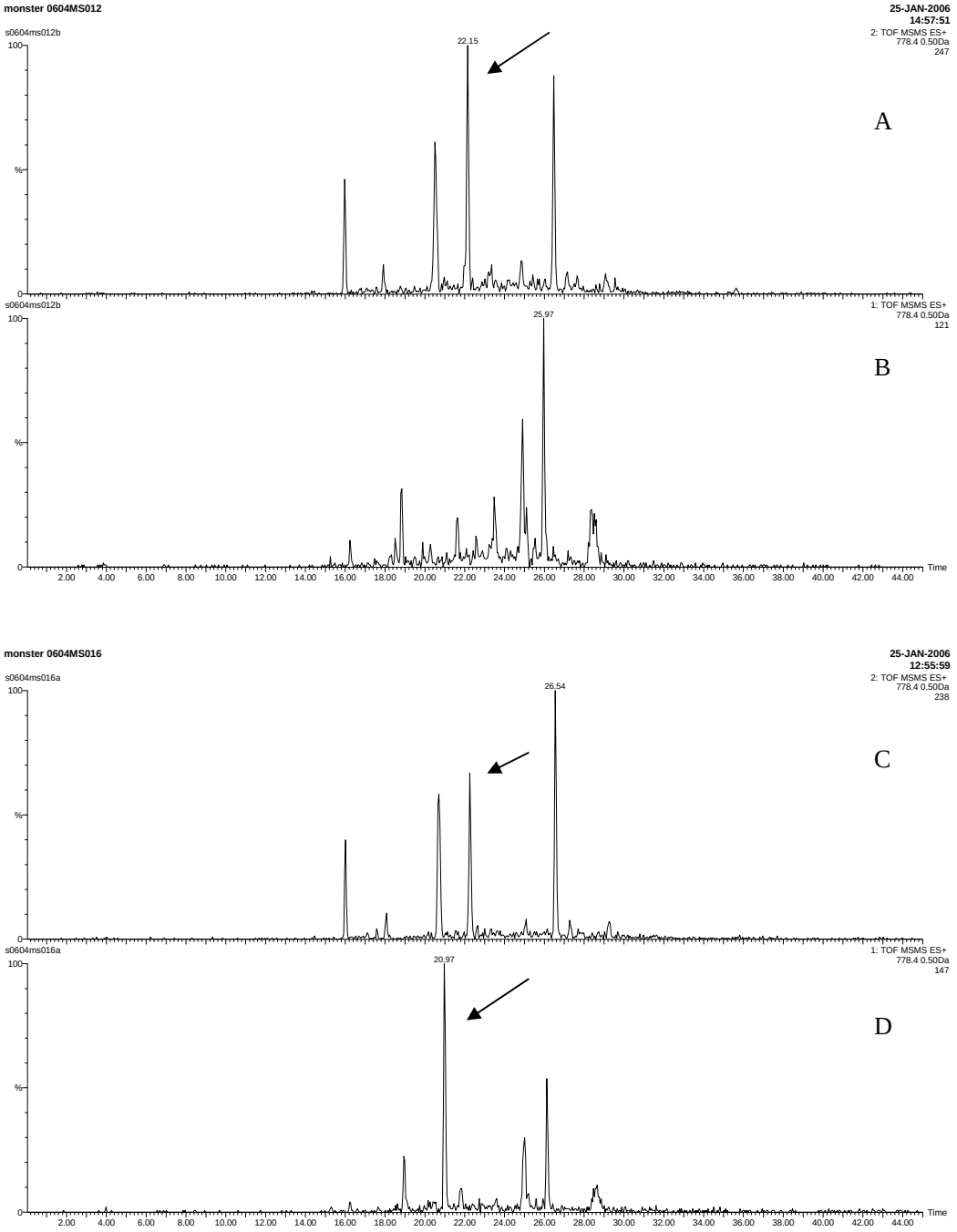


Figure 17. LC tandem MS analysis of FGES(EMPA)AGAAS in pepsin digest of HuBuChE isolated from human plasma; comparison of blank sample (trace A + B) and VX-exposed sample (trace C and D), in presence of internal standard (plasma exposed to d7-sarin).
The arrows indicate the peaks of interest.

Trace A: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 923.4 (MH^+); d_7-sarin (blank)

Trace B: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 902.4 (MH^+); VX (blank)

Trace C: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 923.4 (MH^+); d_7-sarin (exposed)

Trace D: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 902.4 (MH^+); VX (exposed)

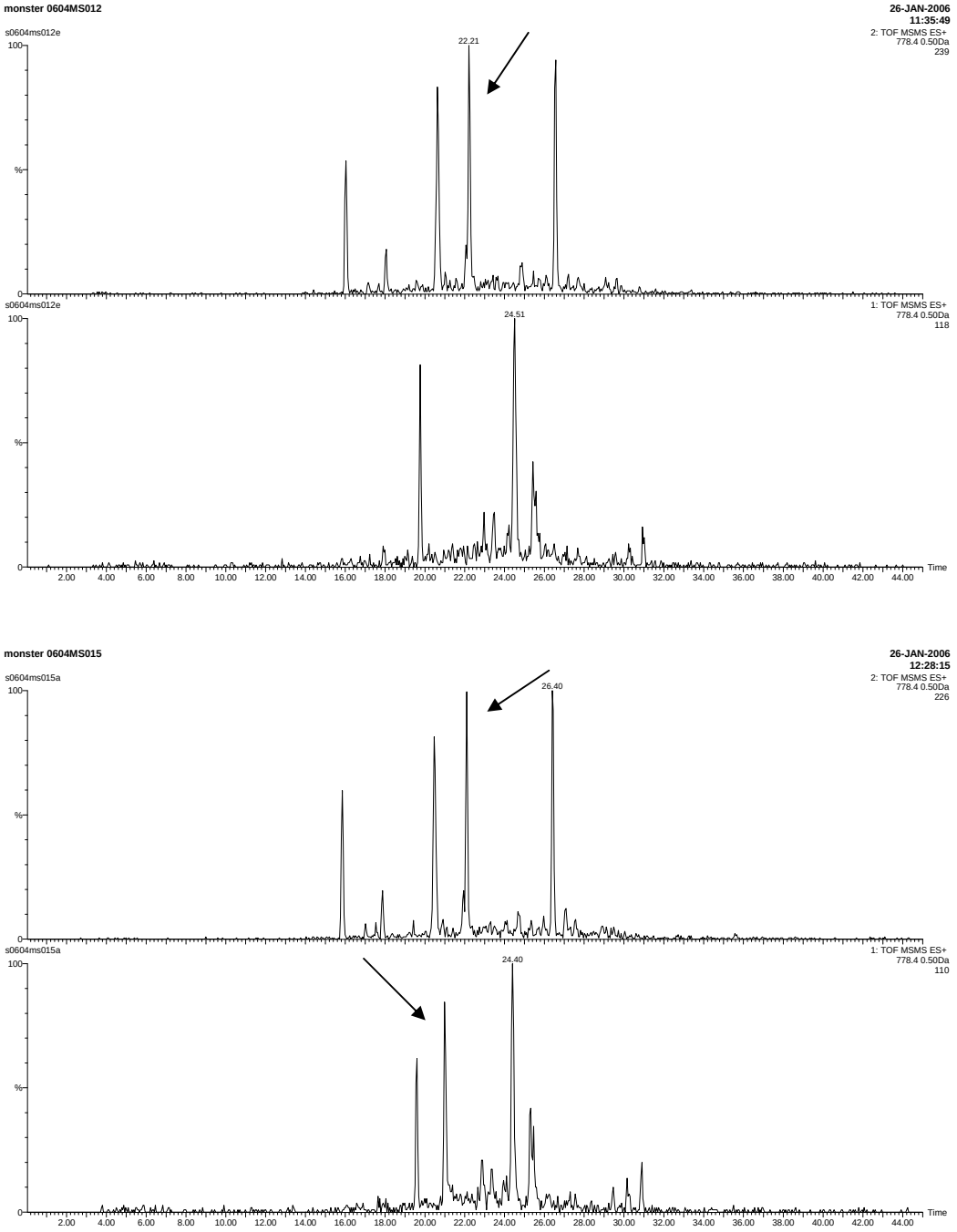


Figure 18. LC tandem MS analysis of FGES(MPA)AGAAS in pepsin digest of HuBuChE isolated from human plasma; comparison of blank sample (trace A + B) and soman-exposed sample (trace C and D), in presence of internal standard (plasma exposed to d7-sarin).
The arrows indicate the peaks of interest.

Trace A: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 923.4 (MH^+); d_7-sarin (blank)

Trace B: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 874.4 (MH^+); soman (blank)

Trace C: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 923.4 (MH^+); d_7-sarin (exposed)

Trace D: Ion chromatogram of fragment ion *m/z* 778.4, after selection of *m/z* 874.4 (MH^+); soman (exposed)

Currently, multiple reaction monitoring (MRM) experiments on our recently acquired triple-quad MS (TSQ Quantum Ultra, ThermoFinnigan) are ongoing, in order to see whether we can improve the sensitivity of the method. A representative example is shown in Figure 19.

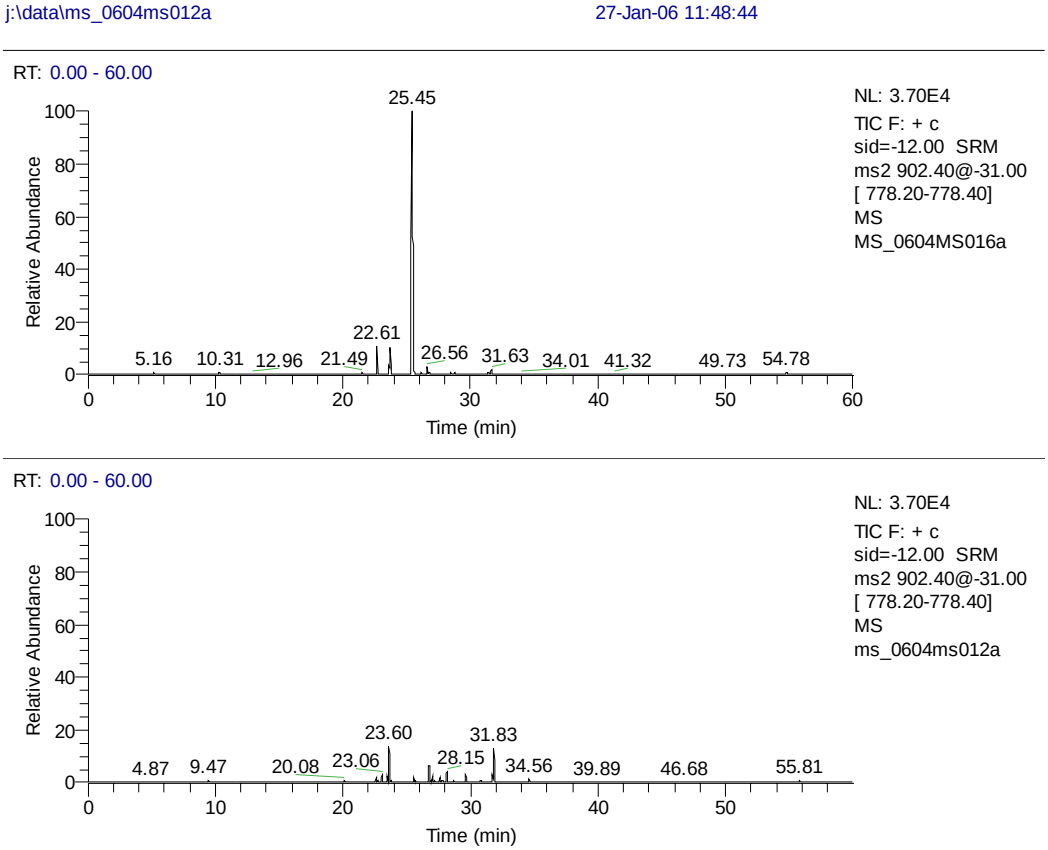
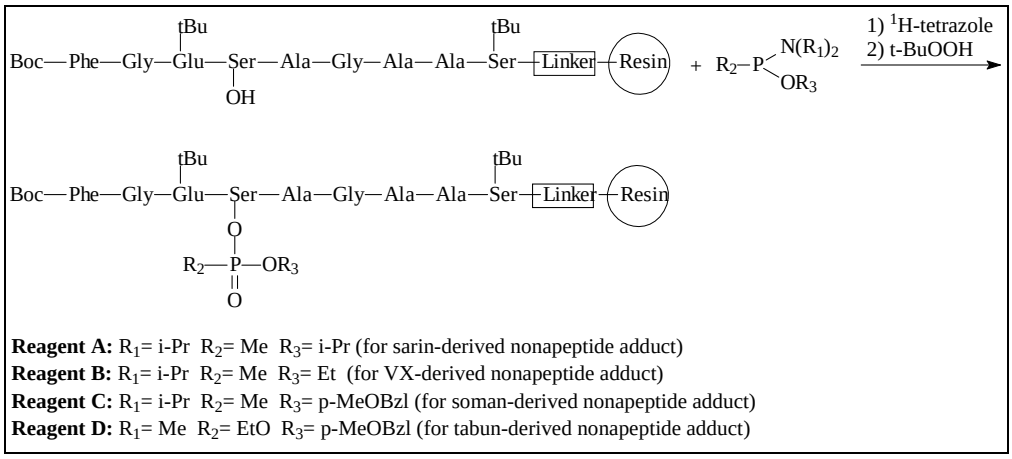


Figure 19. LC tandem MS analysis of FGES(EMPA)AGAAS in pepsin digest of HuBuChE isolated from human plasma; VX-exposed sample (upper trace) and blank sample (lower trace). Multiple reaction monitoring of m/z 902.4 (MH^+) → 778.4

IV.7 Synthesis of reference peptides for cholinesterase assay

The synthesis of four different nonapeptide reference peptides has been performed, following a synthetic route as described in Fidder et al (2002). The peptides will serve as reference compounds for analysis of pepsin digests that are derived from HuBuChE that had been exposed to sarin, soman, VX or tabun. Basically, the synthetic route we followed is outlined in Figure 20. Four different phosphylating reagents A-D were prepared (see Figure 21) and used for reaction with immobilized FGESAGAAS, with S* unprotected to allow modification, i.e., reaction with the phosphylating agent. After oxidation of the phosphite function, followed by cleavage from the resin and deprotection (Figure 22), the peptides were purified to homogeneity by means of reverse phase HPLC. According to this synthesis methodology, milligram amounts of the required nonapeptides could be obtained, which gave satisfactory mass spectrometric data. The peptides could be used for infusion experiments in order to optimize settings of the MS.



Reagent A: R_1= i-Pr R_2= Me R_3= i-Pr (for sarin-derived nonapeptide adduct)
Reagent B: R_1= i-Pr R_2= Me R_3= Et (for VX-derived nonapeptide adduct)
Reagent C: R_1= i-Pr R_2= Me R_3= p-MeOBzl (for soman-derived nonapeptide adduct)
Reagent D: R_1= Me R_2= EtO R_3= p-MeOBzl (for tabun-derived nonapeptide adduct)

Figure 20. Solid phase synthesis of phosphylated nonapeptide reference compounds

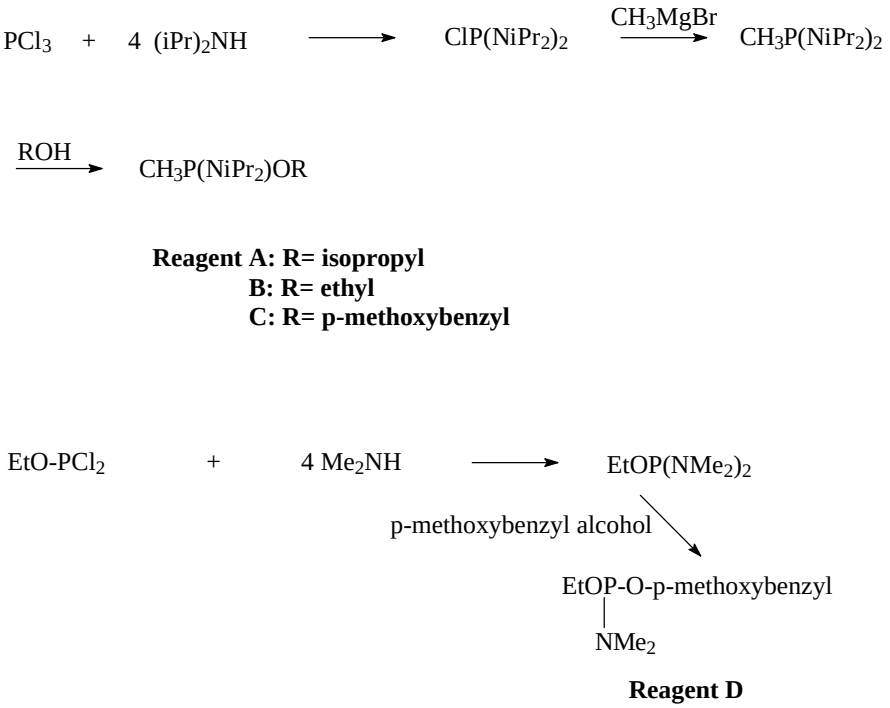


Figure 21. Synthesis of phosphylating reagents required for on resin phosphylation of HuBuChE nonapeptide derivatives.

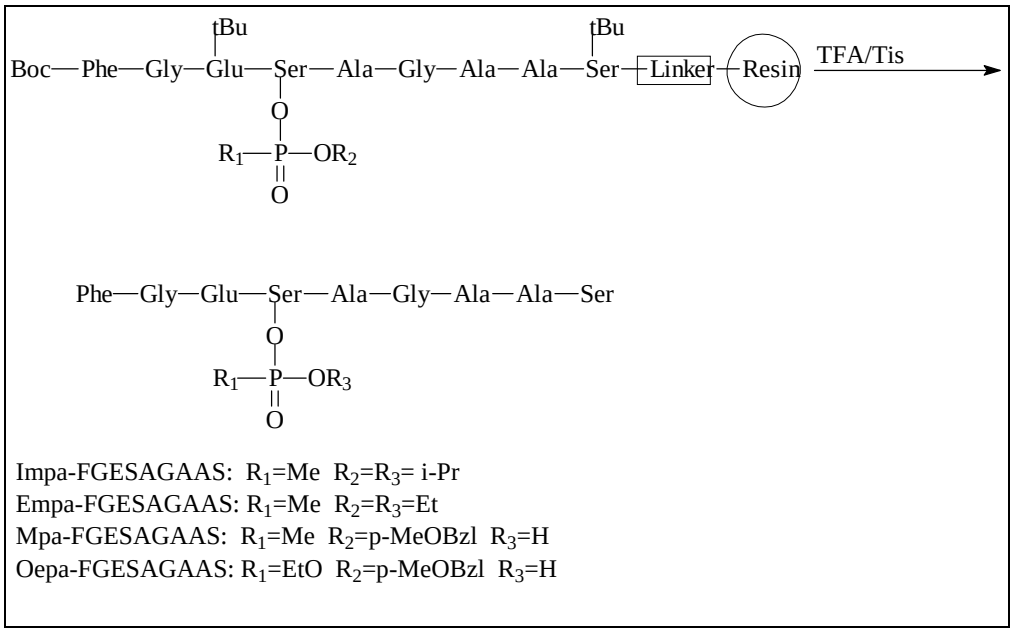


Figure 22. Cleavage from resin and concomitant deprotection of phosphylated nonapeptides

Various MS spectra of the four reference peptides are given in Figures A1-A4 of the annex.

IV.8 Development of a generic assay for OP biomonitoring

Background

In recent years (Noort *et al*, 2002) we have developed a number of assays in order to detect exposure to nerve agents. The fluoride reactivation method is based on the principle that upon incubation of phosphylated binding sites (for example HuBuChE in plasma) with a large excess of fluoride ions, the phosphyl moiety is quantitatively converted into the corresponding phosphono-or phosphorofluoridate (Degenhardt *et al*, 2004). The latter can be isolated by solid phase extraction on a C18 cartridge and quantitated by GC/NPD or GC/MS. The other method is based on mass spectrometric determination of specific peptide adducts that result after pepsin digestion of adducted HuBuChE (Fidder *et al*, 2002). This method surpasses the limitations of the fluoride-reactivation method since it can deal with HuBuChE inhibited with organophosphates that cannot be reactivated or that rapidly age (e.g. soman).

One of the problems with both assays for assessment of organophosphate exposure is that one has to know in advance for which type of nerve agent to screen for during mass spectrometric analysis. It is possible to perform parent ion scan, but this will be at the cost of sensitivity, which means that lower degrees of inhibition cannot be detected anymore. Therefore a generic mass spectrometry-based method for detection of phosphylated HuBuChE is highly needed. In this respect, our attention was raised by a general method which is used for the detection of phosphorylation sites in the field of proteomics (e.g., Oda *et al*, 2001). According to this method, proteins are treated with mild base in order to eliminate the phosphate function, resulting in the formation of a dehydroalanine residue in the protein. Subsequently, the thus formed dehydroalanine residue is subjected to a Michael addition with a simple or functionalized thiol or amine. We here report that this methodology, albeit in a slightly modified form, can be applied to nerve agent-inhibited HuBuChE and that the result is irrespective of the agent used (see Figure 23).

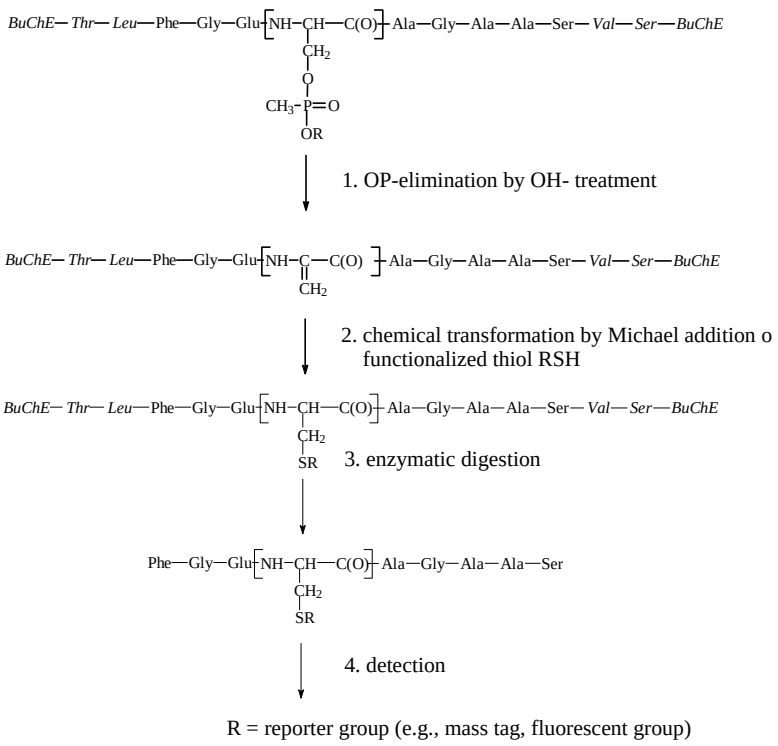


Figure 23. Initial strategy towards generic assay for diagnosis of exposure to OP compounds.

Synthetic FGE(p)SAGAAS was used to discover the most optimal conditions for introduction of a nucleophilic group like a thiol or amine. We first used ethanethiol as nucleophile since it has been used in the field of proteomics. It appeared that performing the reaction in $Ba(OH)_2$ (100 mM) and a nucleophile (50 mM) for 1 h at 37 ºC was most suitable. Longer reaction times would result in hydrolysis of non-phosphorylated serine residues to dehydroalanine, resulting in false positives.

Next, purified HuBuChE was used in order to detect the possibility of a generic assay. Three different nerve agents (VX, sarin, soman) were used to inhibit a portion of purified HuBuChE. The inhibited enzymes were subjected to alkaline hydrolysis like described before, followed by Michael addition of ethanethiol, resulting in all cases in the same S-ethyl FGECAGAAS (see Figures 24 and 25).

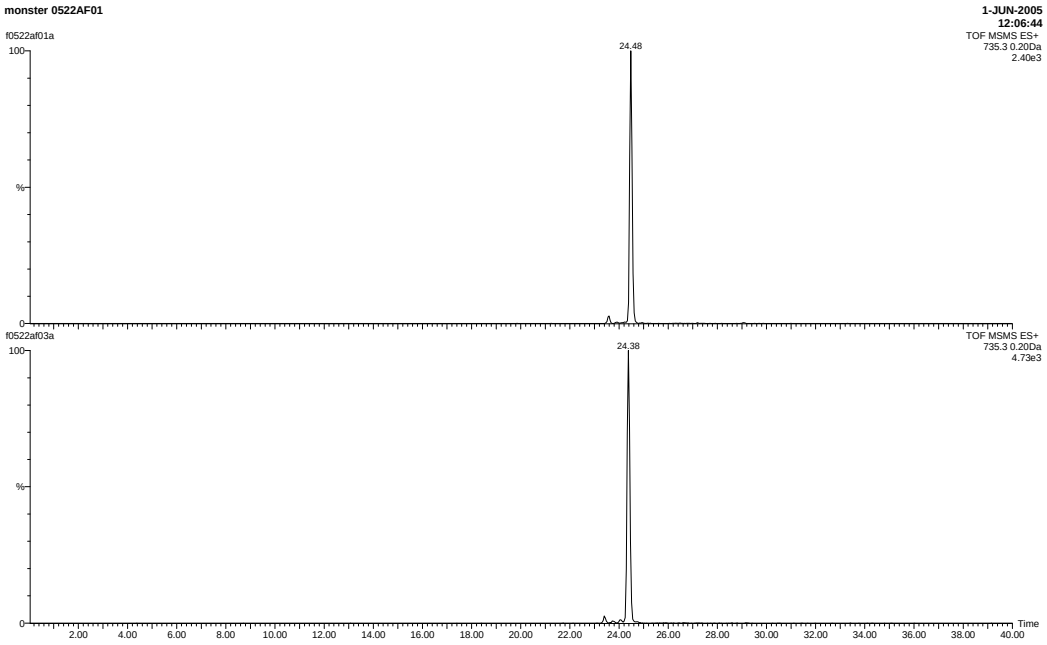


Figure 24. Ion chromatogram of m/z 735.3 of FGE(S-ethyl)CAGAAS obtained after processing pepsin digest of purified HuBuChE with $Ba(OH)_2$ and EtSH. Upper panel: sample inhibited with VX. Lower panel: sample inhibited with soman. In the first MS, the ion with m/z 840.3 (MH^+) had been selected.

The drawback of using ethanethiol, unfortunately, was that the derived S-ethyl nonapeptide lies in a rather "crowded" region of the chromatogram. Furthermore, the degree of fragmentation of the particular S-ethyl nonapeptide is very high (see Figure 25).

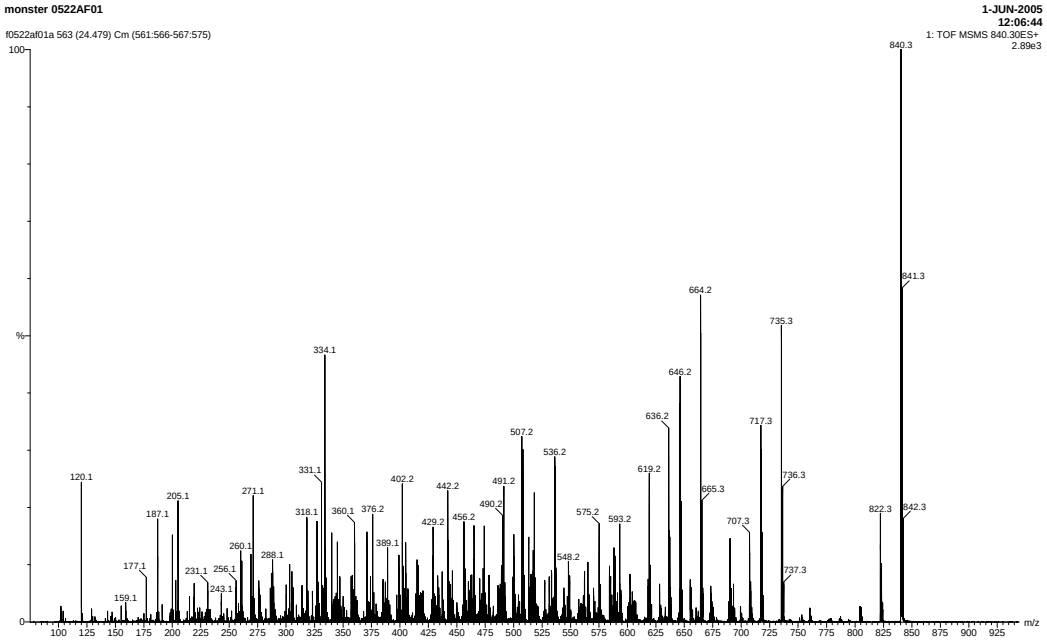


Figure 25. Product ion spectrum of molecular ion MH+ (*m/z* 840.3) of FGE(S-ethyl)CAGAAS obtained after processing pepsin digest of purified HuBuChE with $Ba(OH)_2$ and EtSH.

Again, synthetic FGE(p)SAGAAS was used to evaluate the use of nucleophiles other than ethanethiol. See Figure 26 for a list of nucleophiles that were used. The cysteamine derivatives were prepared in our laboratory. It appeared that the nonapeptide that results by using 2(3-aminopropylamino)ethanol (H) has both favorable chromatographic properties and mass spectrometric properties; a nice MH_2^{2+} fragment could be observed (see Figures 27 and 28).

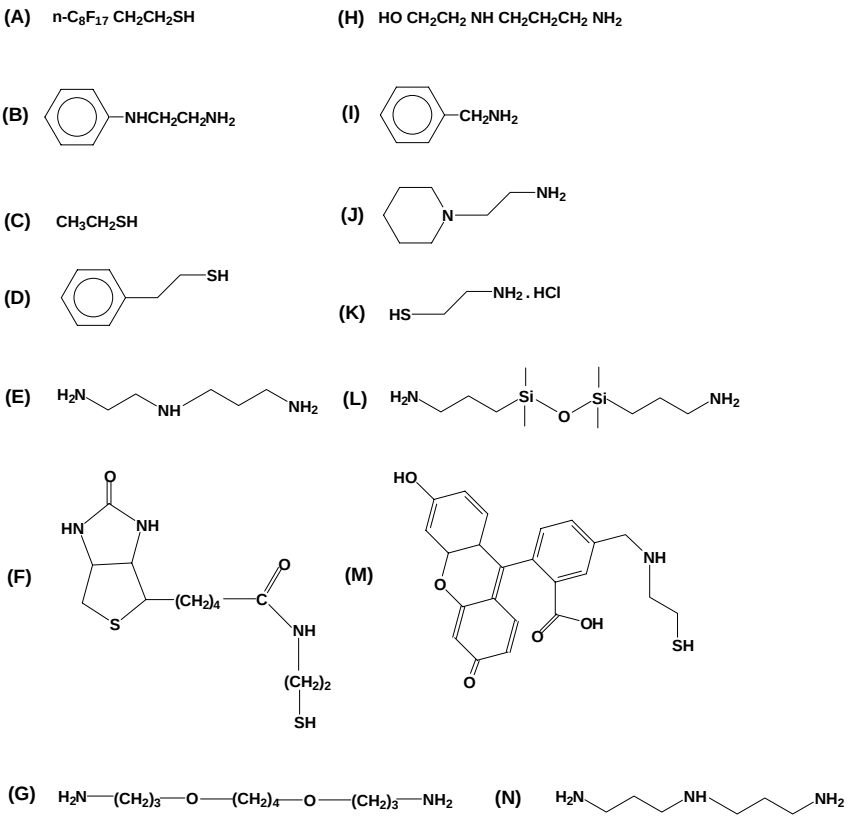


Figure 26. Structures of nucleophiles used for modification of FGE(p)SAGAAS residues after alkaline hydrolysis.

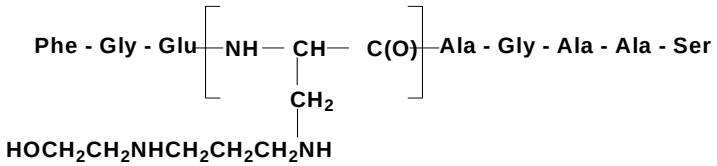


Figure 27. Structure of the modified nonapeptide FGEXAGAAS, with X the converted phosphoserine residue, as analyzed after conversion of the phosphylated serine residue with 2(3-aminopropylamino)ethanol by means of alkaline hydrolysis/Michael addition.

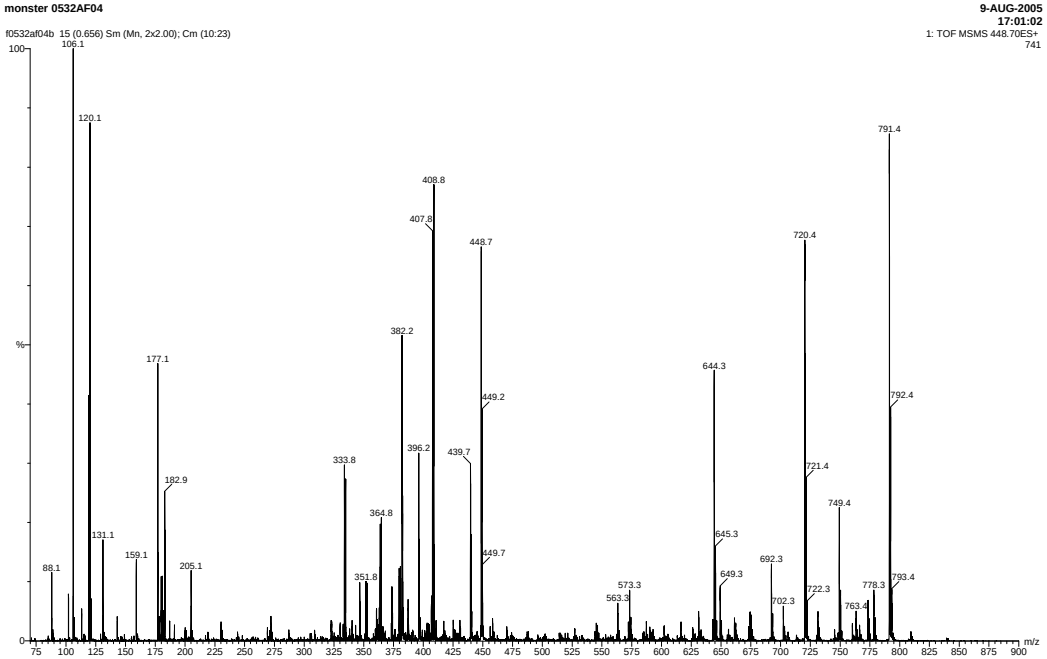


Figure 28. Product ion spectrum of m/z 448.7 (MH_2^{2+}) resulting from conversion of synthetic FGE(p)SAGAAS by alkaline hydrolysis and concomitant reaction with $HOCH_2CH_2NHCH_2CH_2CH_2NH_2$

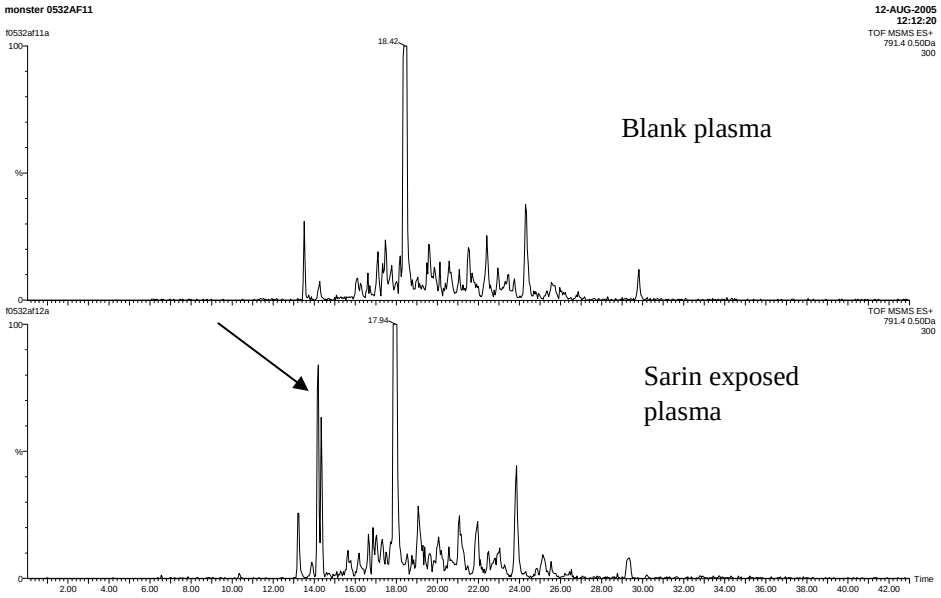


Figure 29. Ion chromatogram of *m/z* 791.4 in processed pepsin digests of isolated HuBuChE from plasma samples. The upper panel represents the ion chromatogram of a processed digest from blank plasma. The lower panel represents the ion chromatogram of a digest from plasma, that had been exposed to sarin. Both digests were subjected to modification with 2(3-aminopropylamino)ethanol, under the agency of $Ba(OH)_2$. The arrow indicates the peak of the modified FGEXAGAAS. In the first MS *m/z* 448.7 (MH_2^{2+}) was selected.

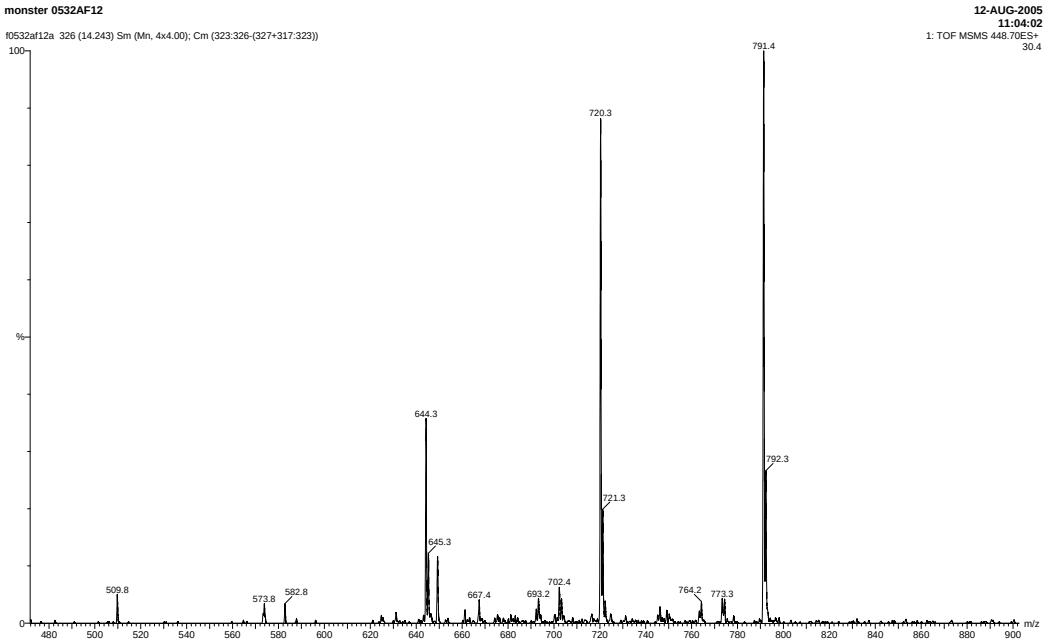


Figure 30. Part of the product ion spectrum of molecular ion MH_2^{2+} (m/z 448.7) resulting from conversion of FGE(p)SAGAAS by alkaline hydrolysis and concomitant reaction with $HOCH_2CH_2NHCH_2CH_2CH_2NH_2$, after processing of a pepsin digest of HuBuChE isolated from a plasma sample that had been exposed to sarin.

It appeared that the direct alkaline hydrolysis and concomitant introduction of the selected nucleophile of HuBuChE was troublesome, probably due to precipitation of various proteins in the alkaline matrix. We decided to perform the pepsin digestion of isolated HuBuChE prior to the combined alkaline treatment/Michael addition. This slightly alternative approach proved to be viable (see Figures 29 and 30). Interestingly, two peaks were observed, which can be explained by the fact that due to the formation of a dehydroalanine residue and subsequent attack of the nucleophile on the double bond, the resulting amino acid loses its chiral integrity. For future experiments, it is planned to remove excessive $Ba(OH)_2$ and nucleophile by means of SPE on Seppak C18, prior to the actual mass spectrometric analysis.

It is envisaged that the desired reference standard can be synthesized through the corresponding phosphononapeptide, as described above. A well-defined amount of this reference standard will be prepared in the next grant period.

The reaction scheme as depicted in Figure 31 will be used for optimization of the approach, as is to be performed in the next grant period. Especially, attention will be paid to improvement of the reproducibility of the assay.

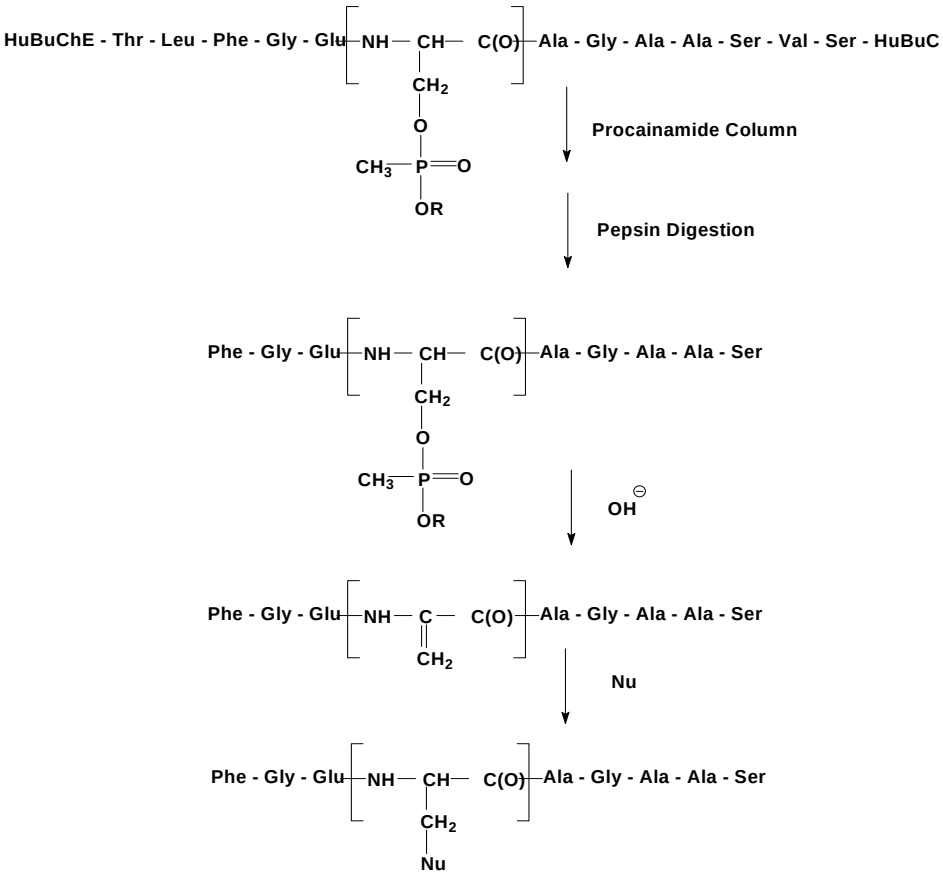


Figure 31. Reaction sequence to convert phosphylated HuBuChE into a modified nonapeptide

Annex to Results section; mass spectrometric data of synthetic phospho-nonapeptides

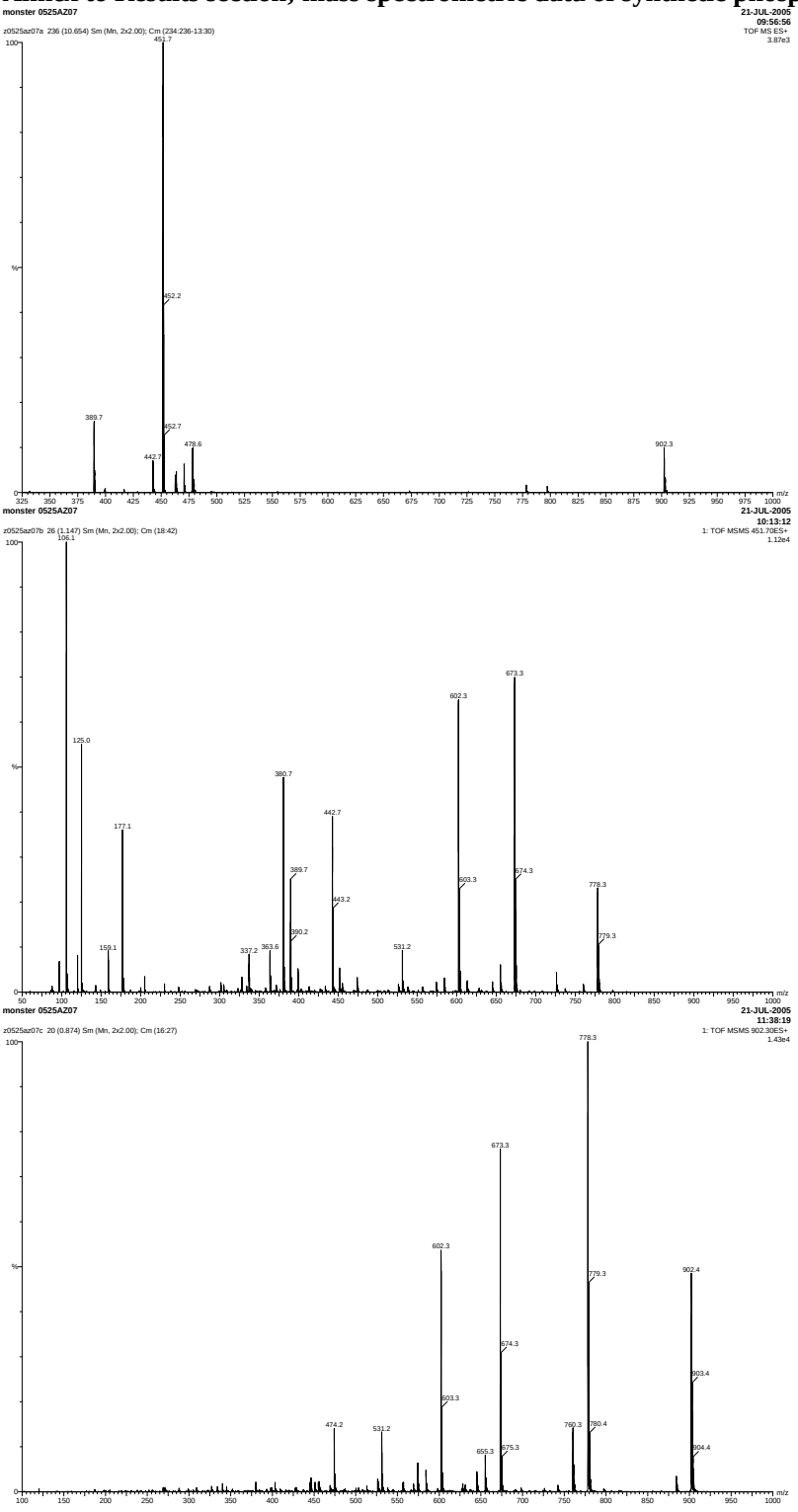


Figure A1. Mass spectra of synthetic FGES(EMPA)AGAAS, i.e., the nonapeptide derived from VX-inhibited HuBuChE. Upper trace: Mass spectrum after flow injection. Middle trace: product ion spectrum of *m/z* 451.7 (MH_2^{2+}). Lower trace: product ion spectrum of *m/z* 902.3 (MH_2^{2+})

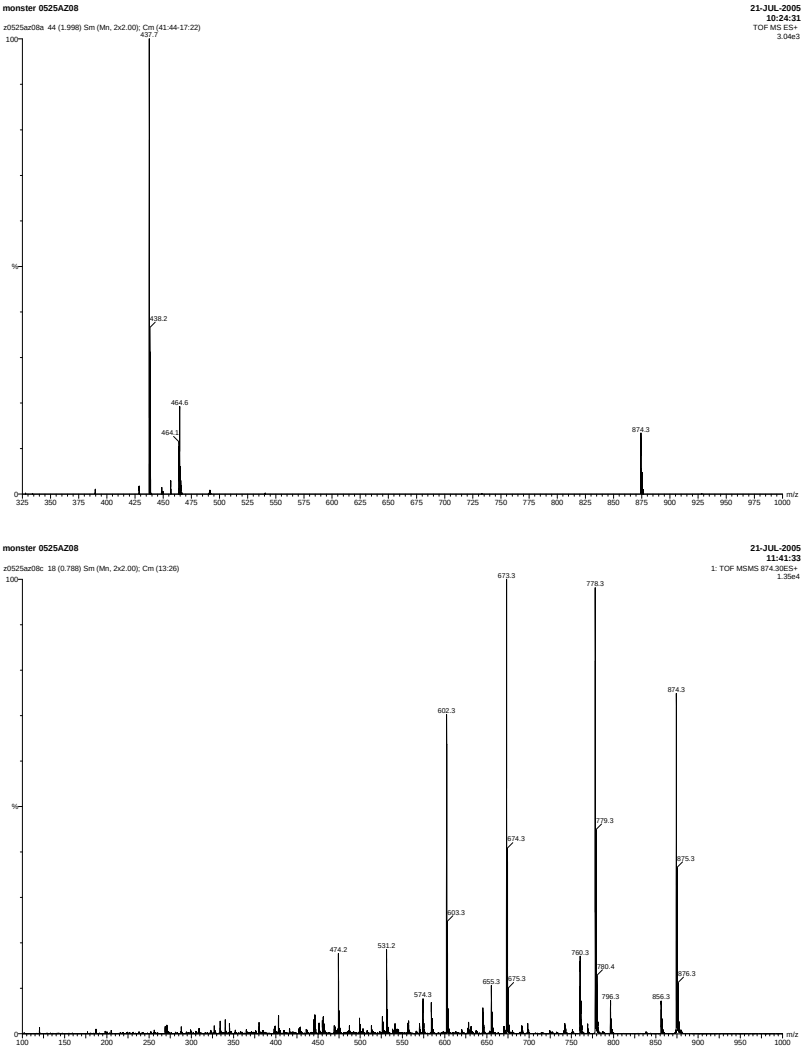


Figure A2. Mass spectra of synthetic FGES(MPA)AGAAS, i.e., the nonapeptide derived from aged soman-inhibited HuBuChE. Upper trace: Mass spectrum after flow injection. Lower trace: Product ion spectrum of *m/z* 874.3 (MH^+).

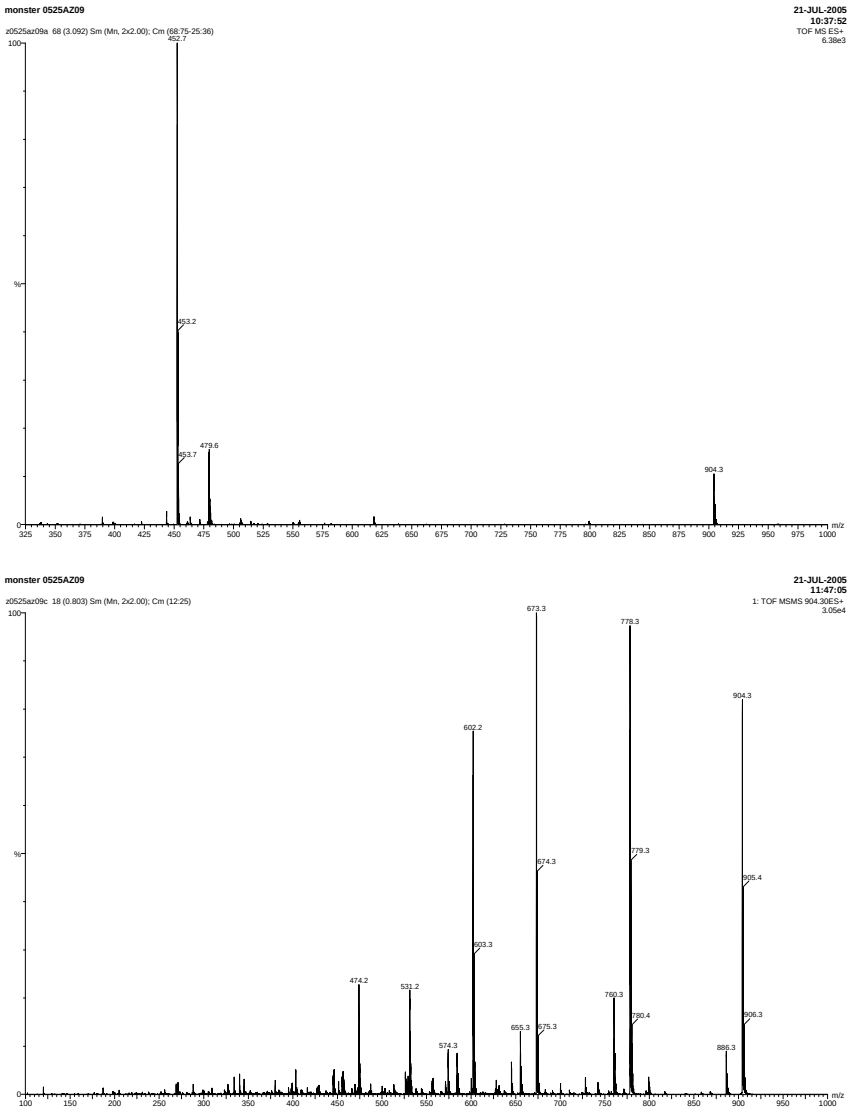


Figure A3. Mass spectra of synthetic FGES(O-ethylphospho)AGAAS, i.e., the presumed nonapeptide derived from tabun-inhibited HuBuChE. Upper trace: Mass spectrum after flow injection. Lower trace: product ion spectrum of m/z 904.3 (MH^+).

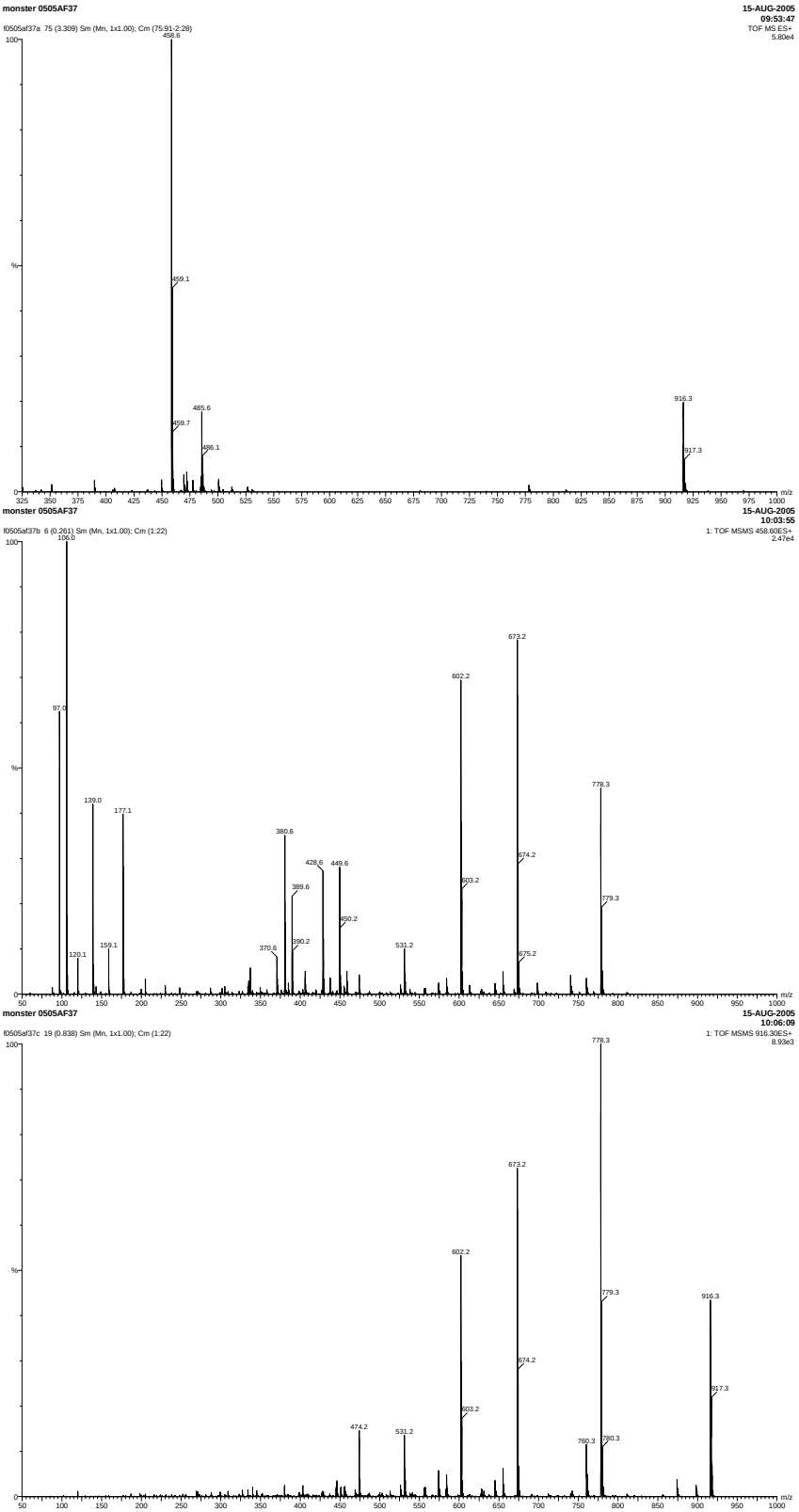


Figure A4. Mass spectra of synthetic FGES(IMPA)AGAAS, i.e., the nonapeptide derived from sarin-inhibited HuBuChE. Upper trace: Mass spectrum after flow injection. Middle trace: product ion spectrum of *m/z* 458.6 (MH_2^{2+}). Lower trace: product ion spectrum of *m/z* 916.3 (MH^+).

V DISCUSSION

Method transfer

Several methods have been transferred to CDC during this grant period. In general, due to the availability of the reference compounds, the methods could relatively be easily be set up. In general, it is recommended that the mass spectrometers should be fine-tuned a few weeks in advance with the synthetic reference standards.

During performance of the work, several relevant observations/recommendations were made:

Modified Edman degradation

- It seems that LC tandem MS analysis of the pentafluorophenylthiohydantoin obtained after modified Edman degradation of sulfur mustard-alkylated globin is not very sensitive. However, it should be recognized that only 10 mg of globin was used (samples were split after modified Edman degradation). Since the traces for the various MRM transitions are quite clean, it might be advantageous to use more globin material (e.g. 50 mg) in order to detect lower exposure levels.
- The samples prepared by modified Edman degradation at TNO could be analyzed satisfactorily; it turns out that the sensitivity of the TSQ7000 instrument at CDC is higher than the sensitivity of the HP5973 MSD at TNO.
- Some of the samples prepared during the method transfer gave deviating results. A number of reasons can be given for this:
    - Instead of concentrating the samples by centrifugal evaporation, as described in the original procedure, the samples were concentrated by heating under a stream of nitrogen. This might result in a significant loss of analyte. It is recommended not to deviate from the standard procedure.
    - Working with relatively high level reference standards during sample preparation of low level exposed globin samples can cause severe problems.
- GC-NICI tandem MS might result in cleaner ion chromatograms and increased sensitivity.

Phosgene method

- It is recommended to use a similar column (PepMap C18, 15 cm x 1 mm; 3 mm) as used at TNO for future analyses
- It is recommended to dissolve synthetic reference standard in a mixture of water/acetonitrile, containing HCOOH or TFA; dilution with water containing HCOOH or TFA
- In order to speed up the method: try to replace the dialysis step using dialyis cassettes by washing on molecular weight cut-off filters. According to the manufacturers instructions, the filters are compatible with 6 M guanidine buffers. In addition, it might be worthwhile to try to do the tryptic digestion on the molecular weight cut-off filter itself.

Lewisite method

- Use 4 mL vials for collecting the Seppak C18 eluate; these vials should fit in a rotor for the Eppendorf Vacufuge
- Use an adjustable heat gun to concentrate the sample volume of 3 mL until an amount of approx 1 mL has been obtained, in order to avoid “bumping” during the concentration step in a vacuum concentrator.

- In order to facilitate the manipulations during extraction after introduction of the HFB group, it is recommended to add some extra toluene (0.5 ml) after performance of the derivatization reaction. After extraction and drying, the toluene layer may be concentrated.

Rapid assay for OP biomonitoring based on LC-tandem MS analysis of pepsin digests of HuBuChE

The pepsin digestion of HuBuChE is a powerful method for diagnosis of an exposure to an organophosphate. In the current grant period, the isolation of HuBuChE from plasma has been further examined. We succeeded in a high-yield isolation of HuBuChE by using only 2 ml of procainamide gel; subsequent pepsin digestion of the crude protein material afforded a digest that could readily be analyzed for the presence of the phosphylated nonapeptide. Attempts to further improve the isolation of HuBuChE from the matrix are ongoing.

Furthermore, a methodology for the preparation of various phosphylated nonapeptides, derived from HuBuChE inhibited with VX, sarin, somand and tabun, has been worked out; the peptides are now available as reference compound in milligram quantities.

Generic assay for OP biomonitoring

We succeeded in developing a novel and generic assay for OP biomonitoring, that is based on pepsin digestion of HuBuChE, followed by base-catalyzed elimination of the phosphyl moiety and subsequent Michael addition of a suitable nucleophile. This results in one common modified nonapeptide, that can be analyzed by means of LC tandem MS. The availability of a phosphylated nonapeptide as model compound significantly helped in finding the most suitable nucleophile, *i.e.,* 2-(3-aminopropylamino)ethanol. It turned out that the phosphyl elimination/Michael addition reaction sequence gave the most reproducible results on the peptide level, which means that the pepsin digestion has to be carried out first. This strategy has the additional advantage that after splitting the sample after pepsin digestion, a two-step approach can be followed. First, the generic method can be used for an intitial screening of samples and after finding a positive sample, the pepsin digest can be analyzed in a more specific way as described in paragraph, in order to unravel the specific nature of the OP inhibitor. The method is still under development; the lowest observable exposure level will be determined in the next grant period.

VI KEY RESEARCH ACCOMPLISHMENTS OBTAINED IN THIS GRANT PERIOD

1. Reference materials for the modified Edman degradation of sulfur mustard-modified hemoglobin have been prepared and shipped to CDC.
2. The method for modified Edman degradation of sulfur mustard-modified hemoglobin has been transferred to CDC.
3. Reference materials for the method for diagnosis of exposure to Lewisite have been prepared and shipped to CDC.
4. The method for diagnosis of exposure to Lewisite has been transferred to CDC.
5. The method for diagnosis of exposure to phosgene has been optimized and transferred to CDC.
6. Reference materials for the method for diagnosis of exposure to phosgene have been prepared and shipped to CDC.
7. The albumin tripeptide assay has further been improved.
8. Reference materials for the albumin tripeptide assay have been shipped to CDC.
9. The rapid assay for determination of exposure to nerve agents, based on pepsin digestion of HuBuChE, has been optimized.
10. A methodology for the solid phase synthesis of phosphylated nonapeptides has been developed.
11. Various reference standards for the rapid assay for determination of exposure to nerve agents have been prepared.
12. A generic method for detection of covalently modified HuBuChE has been developed.

VII REPORTED OUTCOMES (from beginning of cooperative agreement)

Publications

NOORT, D., FIDDER, A., HULST, A.G., and LANGENBERG, J.P. (2002) Low level exposure to sulfur mustard: development of an SOP for analysis of albumin and/or hemoglobin adducts. Proceedings of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

NOORT, D., BENSCHOP, H.P. and BLACK, R.M. (2002) Biomonitoring of exposure to chemical warfare agents. A review. Toxicol. Appl. Pharmacol. **184**, 116-126.

VAN DER SCHANS, G.P., NOORT, D., MARS-GROENENDIJK, R.H., FIDDER, A., CHAU, L.F., DE JONG, L.P.A. and BENSCHOP, H.P. (2002) Immunochemical detection of sulfur mustard adducts with keratins in the stratum corneum of human skin. Chem. Res. Toxicology **15**, 21-25.

VAN DER SCHANS, G.P., NOORT, D., MARS-GROENENDIJK, R.H., FIDDER, A. and LANGENBERG, J.P. (2002) Immunochemical detection of sulfur mustard adducts with keratins in the stratum corneum of human skin. Proceedings of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H., NOORT, D., and LANGENBERG, J.P. (2002) Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Proceedings of the meeting of NATO TG-004, November 2002, Oslo, Norway.

NOORT, D., FIDDER, A., BENSCHOP, H.P., DE JONG, L.P.A. and SMITH, J.R. Procedure for monitoring exposure to sulfur mustard based on modified Edman degradation of globin. J. Anal. Toxicol., **28**, 311-315, 2004.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R., DE JONG, L.P.A., BENSCHOP, H.P. and NOORT, D. Standard operating procedure for immunoslotblot assay for analysis of DNA-sulfur mustard adducts in human blood and skin. J. Anal. Toxicol., **28**, 316-319, 2004.

NOORT, D., FIDDER, A., HULST, A.G., WOOLFITT, A.R., ASH, D., and BARR, J.R. Retrospective detection of exposure to sulfur mustard: Improvements on an assay for liquid chromatography – tandem mass spectrometry analysis of albumin/sulfur mustard adducts. J. Anal. Toxicol., **28**, 333-338, 2004.

NOORT, D., FIDDER, A., DE KANT, S., and HULST, A.G. Low Level Exposure to Sulfur Mustard: Development of an SOP for Analysis of Albumin Adducts. Proceedings of the Bioscience Review 2004, in press.

BLACK, R.M., and NOORT, D. (2005) Methods for retrospective detection of exposure to toxic scheduled chemicals. Part A: Analysis of free metabolites, pp. 403-431. In “Chemical Weapons Convention Chemicals Analysis; sample collection, preparation and analytical methods” Ed. M. Mesilaakso; John Wiley & Sons, Chichester, UK.

NOORT, D., and BLACK, R.M. (2005) Methods for retrospective detection of exposure to toxic scheduled chemicals. Part B: Mass spectrometric and immunochemical analysis of covalent adducts to proteins and DNA, pp 433-451. In "Chemical Weapons Convention Chemicals Analysis; sample collection, preparation and analytical methods" Ed. M. Mesilaakso; John Wiley & Sons, Chichester, UK.

VAN DER SCHANS, G.P., MARS-GROENENDIJK, R.H, BIKKER, F.J. and NOORT, D. (2005) Immunochemical Procedures for Simple Analysis of Exposure to Sulfur Mustard. Contribution to book entitled: "Medical Aspects of Chemical and Biological Terrorism"; "Chemical terrorism and traumatism", edited by Christophor Dishovsky and Professor Alexander Monov, Publ. house of the Union of Scientists in Bulgaria. (in press).

Abstracts

NOORT, D., FIDDER, A., HULST, A.G., and LANGENBERG, J.P. (2002) Low level exposure to sulfur mustard: development of an SOP for analysis of albumin and/or hemoglobin adducts. Book of abstracts of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

VAN DER SCHANS, G.P., NOORT, D., MARS-GROENENDIJK, R.H., FIDDER, A. and LANGENBERG, J.P. (2002) Immunochemical detection of sulfur mustard adducts with keratins in the stratum corneum of human skin. Book of abstracts of the 2002 Medical Defense Bioscience Review, June 2002, Hunt Valley, MD, USA.

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Development of immunochemical assays for detection of sulfur mustard-adducts with proteins. Presented by G.P. Van der Schans at meeting of NATO TG-004, November 2002, Oslo, Norway.

Use of LC tandem MS techniques in identification and sensitive detection of covalent adducts of xenobiotics with proteins". Presented by D. Noort at "8th European ISSX Meeting", Dijon, France. April 27 – May 1, 2003.

Methods for biomonitoring of individuals (involved by destruction of CW's) who may be contaminated with low concentrations of sulfur mustard, lewisite, phosgene and OP's. Presented by G.P. Van der Schans at meeting of CEPA IEX 13.11, Destruction of old chemical munitions, April 2004, Civitavecchia, Italy.

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VIII CONCLUSIONS

1. Several methods for diagnosis of exposure to scheduled compounds could be successfully transferred to CDC.
2. The method for modified Edman degradation of sulfur mustard modified hemoglobin can also be performed by using LC tandem MS, although the method is then less sensitive.
3. The first attempts for semi-automation of the assay for diagnosis of exposure to phosgene proved to be successful.
4. The rapid assay for detection of HuBuChE inhibitors has been converted into a much less laborious method.
5. The developed generic assay is a powerful method that enables the detection of covalently modified HuBuChE.
6. The developed methodology for synthesis of phosphylated nonapeptides derived from HuBuChE enables the milligram-scale production of specific reference standards.

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XI LIST OF PERSONNEL RECEIVING PAY UNDER THIS COOPERATIVE AGREEMENT

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